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# **Biological Functions of Novel Mitochondrial Proteins**

**Liang Wang**

ACADEMIC DISSERTATION

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Cover image: Transmission electron microscopy image of mitochondrial ultrastructure

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*To my beloved family  
and the most precious people in my life*

## TABLE OF CONTENTS

<b>LIST OF ORIGINAL PUBLICATIONS .....</b>	<b>1</b>
<b>ABBREVIATIONS .....</b>	<b>2</b>
<b>ABSTRACT .....</b>	<b>6</b>
<b>REVIEW OF THE LITERATURE.....</b>	<b>8</b>
1. Introduction to mitochondria .....	8
2. Mitochondrial origin.....	8
3. Mitochondria as a target for cancer therapy .....	9
4. Mammalian mitochondrial ultrastructure .....	11
4.1 Mitochondrial outer membrane .....	12
4.2 Mitochondrial intermembrane space .....	12
4.3 Mitochondrial inner membrane .....	13
4.4 Matrix .....	14
5. Mitochondrial protein import pathways .....	15
5.1 Presequence pathway to the matrix and mitochondrial inner membrane .....	15
5.2 Carrier pathway to the mitochondrial inner membrane .....	15
5.3 Oxidative folding pathway of the intermembrane space .....	16
5.4 $\beta$ -barrel pathway of the mitochondrial outer membrane .....	16
5.5 $\alpha$ -helical transmembrane proteins of the mitochondrial outer membrane .....	17
6. Formation and maintenance of crista .....	17
6.1 OPA1 .....	19
6.2 MICOS complex.....	19
6.3 Dimeric ATP synthase .....	20
6.4 BAR domain protein.....	20
7. Mitochondrial dynamics .....	21
7.1 Mitochondrial fission.....	22
7.2 Mitochondrial fusion .....	23
8. Mitochondrial DNA .....	25
8.1 mtDNA structure .....	25
8.2     Replication of mtDNA .....	26
9.    mtDNA-encoded gene expression .....	28
9.1 Transcription of mtDNA.....	29
9.2 Translation of mtDNA-encoded proteins .....	30
<b>AIMS OF THE STUDY .....</b>	<b>35</b>
<b>MATERIALS AND METHODS .....</b>	<b>36</b>
<b>RESULTS AND DISSCUSSION .....</b>	<b>40</b>
10. Cellular localization of FAM92A1 and GTPBP8 .....	40
10.1 Identification of novel proteins FAM92A1 and GTPBP8 .....	40
10.2 FAM92A1 and GTPBP8 are mitochondrial proteins .....	40
10.3 Sub-mitochondrial localization of FAM92A1 and GTPBP8 .....	41

10.4 Mitochondrial import of FAM92A1 and GTPBP8 .....	42
11. Function characterization of FAM92A1 and GTPBP8 .....	44
11.1 Depletion of FAM92A1 and GTPBP8 results in reduced cell proliferation.....	44
11.2 Depletion of FAM92A1 and GTPBP8 increases oxidative stress in cells.....	44
11.3 Loss of FAM92A1 and GTPBP8 impairs mitochondrial respiration.....	44
11.4 Depletion of FAM92A1 and GTPBP8 leads to aberrant assembly of respiratory chain complexes .....	45
11.5 Aberrant level of respiratory complexes in FAM92A1 and GTPBP8 depletion cells was caused by different mechanisms .....	45
12. FAM92A1 regulates mitochondrial morphology and inner membrane architecture .....	45
12.1 FAM92A1 depletion induces abnormal mitochondrial morphology and impairs mitochondrial dynamics .....	45
12.2 Loss of FAM92A1 causes aberrant mitochondrial inner membrane architecture .....	46
13. FAM92A1 binds to negatively charged phospholipids generating positive membrane curvature .....	47
13.1 FAM92A1 forms dimers and binds mitochondrial membranes.....	47
13.2 FAM92A1 preferentially binds to negatively charged phospholipids.....	47
13.3 FAM92A1 sculpts membranes into tubular structure.....	47
14. Membrane binding interface of FAM92A1 .....	48
15. Cellular function of GTPBP8.....	49
15.1 GTPBP8 depletion causes defects in mitochondrial translation .....	49
15.2 GTPBP8 specifically interacts with the large subunit of human mitoribosomes.....	49
15.3 GTPBP8 deletion causes abnormal accumulation of mt-LSU and reduction of mature mitoribosomes.....	49
16. Prickly nanoparticles efficiently inhibit malignant growth of cancer cells via mechanical damage to mitochondria .....	50
<b>FINAL REMARKS AND FUTURE PERSPECTIVES.....</b>	<b>51</b>
<b>ACKNOWLEDGEMENTS .....</b>	<b>52</b>
<b>REFERENCES.....</b>	<b>54</b>

## LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following two publications and one manuscript. The figures and content in these articles are referred to according to the Roman numerals. The articles are re-printed with the kind permission from the publishers.

(I) **Wang, L.**, Yan, Z., Vihinen, H., Eriksson, O., Wang, W., Soliymani, R., Lu, Y., Xue, Y., Jokitalo, E., Li, J., & Zhao, H. (2019). FAM92A1 is a BAR domain protein required for mitochondrial ultrastructure and function. *J Cell Biol*, 218(1), 97-111.

(II) Zhang, H., Liu, D., **Wang, L.**, Liu, Z., Wu, R., Janoniene, A., Ma, M., Pan, G., Baranauskiene, L., Zhang, LL., Cui, W., Petrikaite, V., Matulis, D., Pan, JM., Zhao, H., & Helder A, S. (2017). Microfluidic Encapsulation of Prickly Zinc-Doped Copper Oxide Nanoparticles with VD1142 Modified Spermine Acetalated Dextran for Efficient Cancer Therapy. *Advanced healthcare materials*, 6(11), 1601406.

(III) **Wang, L.**, Yan, Z., Liu, X., Wang, W., Eriksson, O., Varjosalo, M., Li, J., Zhao, H. GTPBP8 is required for mitoribosomal biogenesis and mitochondrial translation. (Unpublished Manuscript)

### The author's contribution

(I) Liang Wang carried out experiments and prepared figure 1, figure 2, figure 3 (A, B, C, E, F, G and H), figure 5 (A, D, E, F, G, H, I, J, K, L), figure S2 (A, B, C, D, E, F, G, H, I, J, K and L) and figure S3 (B, C, E, F, G and H). All statistic data were quantified by Liang Wang and also finished the electron tomography with the help of Vihinen, H. Liang Wang arranged all figures with input from all authors.

(II) Liang Wang performed experiments and prepared figure 7 and figure 8.

(III) Liang Wang carried out all experiments except for figure 5 (A and F) and figure 6C. Liang Wang quantified all data, prepared all figures and wrote the manuscript with Zhao, H.

## ABBREVIATIONS

ATP	Adenosine triphosphate
ATPases	Adenosine triphosphatases
BAR	Bin-Amphiphysin-Rvs
BN-PAGE	Blue-native polyacrylamide gel electrophoresis
CJs	Crista junctions
CL	Cardiolipin
CM	Crista membrane
cryoET	Electron cryotomography
DABCO	1,4-diazabicyclo[2.2.2]octane
DEAD	Asp-Glu-Ala-Asp
DHE	Dihydroethidium
Dyn2	Dynamin 2
DDSs	Drug delivery systems
DOX	Doxorubicin
DRPs	Dynamin-related proteins
Drp1	Dynamin-related protein 1
DUF143	Domain of unknown function 143
EFs	Elongation factors
ER	Endoplasmic reticulum
FCCP	Carbonyl cyanide-p-trifluoromethoxy-phenylhydrazone
FASTKD2	Fas-activated serine-threonine kinase 2
Fe-S	Iron-sulfur
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GRSF1	G-rich sequence binding factor 1
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
GTPBP8	GTP-binding protein 8
HA	Hyaluronic acid
HAP	Hydroxyapatite

HSP	H-strand promoter
HSP60	Heat shock protein 60
IBM	Inner boundary membrane
ICS	Intracristal space
IF3mt	Mitochondrial initiation factor 3
IMS	Intermembrane space
INF2	Inverted formin 2
L-OPA1	Long form of OPA1
LSP	L-strand promoter
MDR	Multiple drug resistance
MELAS	Mitochondrial myopathy, encephalopathy, lactic acidosis, strokes
Mff	Mitochondrial fission factor
Mfn1	Mitofusin 1
Mfn2	Mitofusin 2
MIA	Mitochondrial intermembrane space import and assembly
MIB	Mitochondrial intermembrane space bridge
MICOS	Mitochondrial contact site and cristae organizing system
MiD49	Mitochondrial dynamics proteins 49
MiD51	Mitochondrial dynamics proteins 51
MIM	Mitochondrial inner membrane
MINOS	Mitochondrial inner membrane organizing system
MitoSox	Mitochondrial targeted fluorescent superoxide sensor
MOM	Mitochondrial outer membrane
MOMP	Mitochondrial outer membrane permeabilization
MPP	Mitochondrial processing peptidase
MRM1	Mitochondrial rRNA methyltransferase 1
MRPs	Mitochondrial ribosomal proteins
mRNA	Messenger RNA
MTase	Methyltransferase
mtDNA	Mitochondrial DNA
mtHSP70	Mitochondrial matrix 70 kDa heat shock protein
MTERF1	Mitochondrial termination of factor 1



mt-LSU	Mitochondrial ribosomal large subunit
MTS	Mitochondrial targeting sequence
mtSSB	Mitochondrial single-stranded DNA-binding protein
mt-SSU	Mitochondrial ribosomal small subunit
MTT	Thiazolyl Blue Tetrazolium Bromide
NPs	Nanoparticles
OCR	Oxygen consumption rate
O <sub>H</sub>	Origin of H-strand replication
O <sub>L</sub>	Origin of L-strand replication
OPA1	Optic atrophy 1
OXPHOS	Oxidative phosphorylation
PAM	Presequence translocase-associated motor
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
RNP	RNA-protein complex
PI(4,5)P <sub>2</sub>	Phosphatidylinositol 4, 5-bisphosphate
PINK1	Pten-induced kinase 1
PLGA-PEG	Poly (lactic-co-glycolic acid)- $\beta$ -poly (ethylene glycol)
PTC	Polytidyl transferase center
POL $\gamma$	Polymerase- $\gamma$
POLRMT	Mitochondrial RNA polymerase
ROS	Reactive oxygen species
RF1mt	Mitochondrial release factor
RRF1mt	Mitochondrial ribosome recycling factor
RT-qPCR	Real-time quantitative PCR
rRNA	Ribosomal RNA
SAM	Sorting and assembly machinery
SDM	Strand-displacement model
siRNA	Small interfering RNA
S-OPA1	Short form of OPA1
ssDNA	Single-stranded DNA
TEM	Transmission electron microscopy

TEFM	Mitochondrial transcription elongation factor
TFAM	Mitochondrial transcription factor A
TFB1M	Mitochondrial transcription factor B1
TFB2M	Mitochondrial transcription factor B2
TIM	Translocase of the MIM
TIM22	Translocase of the MIM 22
TIM23	Translocase of the MIM 23
TMRM	Tetramethylrhodamine methyl ester
TOM	Translocase of the MOM
TOM70	Translocase of the MOM subunit of 70 kDa
TRAP1	Tumor necrosis factor receptor-associated protein 1
tRNA	Transfer RNA
VDAC	Voltage-dependent anion channel
Zn-CuO	Zinc-doped copper oxide
$\Delta\Psi_m$	Mitochondrial membrane potential

## ABSTRACT

Mitochondria are a powerhouse for cells and a hub for numerous signaling pathways. Mitochondria are highly dynamic organelles, frequently changing their shape by shifting the balance of fusion and fission. Dysregulation in mitochondrial function or dynamics causes many human diseases. Among all mammalian organelles, mitochondria are unique organelles containing proteins encoded by two genomes. The sub-mitochondrial localization and biological function of 13 mitochondrial DNA (mtDNA)-encoded proteins have been clearly characterized, while many novel nuclear-encoded mitochondrial proteins are yet to be discovered. To comprehensively determine the mechanisms of mitochondrial-related diseases, it is imperative to reveal cellular functions of key mitochondrial proteins essential for mitochondrial biogenesis, structure, function, and dynamics. In this thesis, we have identified two novel nuclear-encoded mitochondrial proteins in mammalian cells: the Bin-Amphiphysin-Rvs (BAR) protein FAM92A1 essential for regulation of mitochondrial membrane ultrastructure, and the guanosine triphosphate (GTP)-binding protein GTPBP8 required for mitochondrial translation. Due to the critical roles of mitochondria in cell signaling and cell survival, mitochondria, especially mitochondrial proteins, are the potential drug targets for treatment of a wide spectrum of diseases, including cancer. Thus, prickly zinc-doped copper oxide (Zn-CuO) nanoparticles (prickly NPs) are designed and synthesized for cancer therapy. Prickly NPs exhibit very effective antiproliferative capability, and the mechanism study shows that prickly NPs induce severe damages to mitochondria and the endoplasmic reticulum (ER), causing cancer cell death.

In paper I, FAM92A1, as a novel BAR domain protein, localizes to the matrix side of the mitochondrial inner membrane (MIM) of U2OS cells through an N-terminal mitochondrial targeting sequence. Loss of FAM92A1 causes a severe disruption to mitochondrial morphology, ultrastructure, and membrane dynamics, impairing organelle bioenergetics. Furthermore, the purified recombinant FAM92A1 protein binds to model membranes through preferential binding to negatively charged phospholipids. After insertion into a lipid bilayer, FAM92A1 transforms spherical liposomes into narrow tubules, verifying its membrane-remodeling activity. Importantly, the aberrant mitochondrial morphology and function caused by depletion of FAM92A1 can be rescued by wild-type FAM92A1, but only partially rescued by the FAM92A1 mutants with defects in membrane binding and remodeling activity. In paper III, a novel GTP-binding protein GTPBP8 localizes to the mitochondrial matrix associated with the mitochondrial inner membrane of U2OS cells. The N-terminal 46aa is indispensable for the mitochondrial localization of GTPBP8. Importantly, GTPBP8 exclusively interacts with the large subunit of mitochondrial ribosome. Genetic knockdown of GTPBP8 causes a significant reduction in the level of mitoribosomes, inducing defects in mitochondrial translation and mitochondrial bioenergetics. In paper II, prickly NPs are synthesized and modified for optimal cancer targeting. The prickly NPs are very effective in inducing cell death of 3T3 and MCF-7 cancer cell lines. The prickly NPs efficiently accumulate in mitochondria, resulting in severe mechanic disruption to mitochondria and inducing cell apoptosis.

Collectively, these studies reveal the biological functions of two previously uncharacterized mitochondrial proteins and an efficient nanoparticle cancer treatment by targeting mitochondria and ER of cancer cells. These new findings elucidate the crucial relation between mitochondrial form and

function, and hence, contribute to establishing the concept of membrane-mediated signaling in mitochondria. Furthermore, mitochondrial gene expression is critical for maintaining cellular homeostasis. Therefore, the mitochondrial proteins and mitochondria per se are valuable potential drug targets for overcoming many mitochondrial diseases, including MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, strokes) syndrome, Parkinson disease, and cancer, among others. Taken together, this study elucidates the cellular functions of novel mitochondrial proteins and applies synthesized nanoparticles targeted to mitochondria for cancer treatment. The findings may open new avenues for developing novel therapeutics strategies for mitochondrial dysfunction-linked human diseases.

# REVIEW OF THE LITERATURE

## 1. Introduction to mitochondria

Mitochondria are semi-autonomous organelles that contain their own genome and protein synthesis machinery. The most important function of mitochondria is adenosine triphosphate (ATP) generation through oxidative phosphorylation (OXPHOS), thereby regulating cellular metabolism in all eukaryotic cells (du Plessis et al. 2015). In addition to their role in energy conversion, mitochondria are also involved in a wide variety of critically important eukaryotic cellular processes, including apoptosis, iron-sulfur (Fe-S) cluster biosynthesis, fatty acid catabolism, lipids, quinone and steroid biosynthesis,  $\text{Ca}^{2+}$  homeostasis, amino acid and nucleotide metabolism, and regulation of cell signaling through reactive oxygen species (ROS), contributing to the development of metabolic disorders such as insulin resistance, diabetes and so on (Litvinova et al. 2015; Newman, He, and Verdin 2012). The pleiotropic roles of mitochondria are matched by their morphological and ultrastructural complexity in response to different physiological conditions (Glytsou et al. 2016). Mitochondria form a dynamic tubular network that is dispersed throughout cells and subject to continuous rearrangements mediated by fusion and fission events. (Westermann 2010). The overall morphology and ultrastructure of mitochondria are determined by two membrane systems, the bordering outer membrane and the inner membrane that surrounds the central matrix space. This organization allows sub-compartmentalization of biochemical processes (Harner et al. 2014). Numerous proteins localized in the inner membrane are involved in maintaining the mitochondrial architecture. Aberrant mitochondrial forms and functions are widely accepted pathogenic mechanisms in a subset of human diseases, including cancer (Barrera et al. 2016), neurodegenerative disease (Johri and Beal 2012), diabetes (Sivitz and Yorek 2010), and cardiomyopathies (El-Hattab and Scaglia 2016).

## 2. Mitochondrial origin

Mitochondria originate from Gram-negative bacterial progenitors that were engulfed by a primordial eukaryotic “host” cell through endosymbiosis more than 1.5-2 billion years ago. Phylogenetic analysis of mitochondrial genes and their genomic organization and distribution indicate that all mitochondria derive from a common ancestral organelle, originating from the integration of a free-living alphaproteobacteria into a host cell related to Asgard Archaea. (Cavalier-Smith 2006; Dolezal et al. 2006; Dyll et al. 2004; Gross and Bhattacharya 2009). The class alphaproteobacteria encompasses well-defined diverse orders: the Rhizobiales, Rhodobacterales, Caulobacterales, Sphingomonadales, Pelagibacterales, Rhodospirillales, and Rickettsiales. Phylogenetic reconstruction suggests mitochondria as emerging either within, or as a sister group to, the Rickettsiales (Lee, Taylor, and Millar 2013; Roger, Muñoz-Gómez, and Kamikawa 2017). Although all mitochondria in different species come from a common alphaproteobacterial ancestor, phylogenetic reconstructions based on mitochondrial DNA (mtDNA) sequences identified enormous variation in genome size, ranging from the tiny apicomplexan mtDNA (6 kbp) to the expansive plant mtDNA (>150 kbp) (S. Anderson et al. 1981). The complete size of human mtDNA is 16,569 base pairs. Despite divergent size, the coding function of mtDNA is relatively conserved. In general,

mtDNA in different species codes genes involved in mitochondrial translation apparatus, electron transport, and oxidative phosphorylation (Bullerwell and Gray 2004; Burger, Gray, and Lang 2003).

How the proto-mitochondrial ancestor invaded and avoided elimination by the host has generated many hypotheses. Phylogenetic studies have found a number of ancestral bacterial genes to have been transferred to the nuclear genome, resulting in reduction and compaction of the mitochondrial genome. The combination of genomics, proteomics, and energy metabolism studies has suggested two fundamentally different endosymbiotic models for the origin of mitochondria. One model is the archezoan scenario: “the host of the proto-mitochondrial endosymbiont was amitochondrial eukaryote, termed archezoan” (Koonin 2010). The archezoan scenario most closely approximates the classical endosymbiont hypothesis of mitochondrial origin (Margulis 1970). The other model is the symbiogenesis scenario: “a single endosymbiotic event took place that involved the uptake of an  $\alpha$ -proteobacterium by an archaeal cell leading to generation of mitochondria,” subsequently followed “by the evolution of the nucleus and compartmentalization of the eukaryotic cell” (Koonin 2010). The best example of the symbiogenesis scenario is the hydrogen hypothesis. The hydrogen hypothesis states that eukaryotes evolved through symbiotic association of an anaerobic, strictly hydrogen-dependent, strictly autotrophic archaebacterium (the host) with a eubacterium (the symbiont) that was able to respire, but generated molecular hydrogen as a waste product of anaerobic heterotrophic metabolism. The host’s dependence upon molecular hydrogen produced by the symbiont is proposed to be the selective principle that forged the common ancestor of eukaryotic cells (Check 2012; Martin and Müller 1998). The fundamental difference between these two models of mitochondrial origin is whether the alpha-proteobacterial endosymbiosis that gave rise to the proto-mitochondria happened at the same time as and was integral to the formation of the eukaryotic cell (symbiogenesis scenario) or occurred subsequent to the formation of a primitive, amitochondriate cell that served as the host and that was already essentially eukaryotic (archezoan scenario) (Check 2012).

Basing on the theory of endosymbiosis, much of the endosymbiont genome was gradually integrated into the nuclear chromosomes. However, the genome still maintains some of its ancestral features such as the double membrane, its own genetic material, and specific translation machinery (Check 2012). The transition from endosymbiotic bacterium to permanent organelle entailed a massive number of evolutionary changes, including the origins of hundreds of new proteins and the corresponding import system, insertion of membrane transporters, integration of metabolism and reproduction, endosymbiotic gene transfer, lateral gene transfer, and the retargeting of proteins. All of these changes occurred incrementally as the endosymbiont and the host became integrated (Roger et al. 2017).

### **3. Mitochondria as a target for cancer therapy**

Mitochondria exert both pivotal roles and lethal functions in physiological and pathological scenarios (Schimmer et al. 2008; Schmitt and Zischka 2018). On one hand, emerging studies have elucidated mitochondrial bioenergetics, biosynthesis, and signaling pathways to be essential for cancer cell growth, tumorigenesis, and metastasis (Feeley et al. 2015; Giampazolias and Tait 2016; Herst, Grasso, and Berridge 2018; Vyas, Zaganjor, and Haigis 2016). On the other hand, mitochondria are a suicidal weapon store for cells and dozens of cell death-involved signal transduction pathways converge on

mitochondria. This characteristic sensitizes them to drug treatment (Brian J. Altman, Zachary E. Stine, and Chi V. Dang 2016; Weinberg and Chandel 2015). In contrast to mitochondria of normal differentiated cells, the structure and function of mitochondria in cancer cells have changed markedly. For example, the metabolic pattern is reprogrammed, which primarily relies on aerobic glycolysis for satisfying energy and building block requirements of cancer cells (Heiden, Cantley, and Thompson 2009; Liberti and Locasale 2016). Basing on these premises, a growing number of mitochondrial-targeted compounds have been developed for efficient cancer therapy (Schmitt and Zischka 2018; Wallace 2012). So far, emerging compounds that induce the overproduction of ROS, increase the permeabilization of mitochondrial membrane, or reprogram mitochondrial metabolic pathway have acquired extraordinary efficacy in cancer therapy (Schmitt and Zischka 2018; Yang et al. 2016).

Despite obvious efficiency in cancer therapy, many anticancer agents face challenges in clinical application such as diverse side-effects for patients, low bioavailability caused by multiple biological barriers, and absence of precise targeting (Carelle et al. 2002; Nussbaumer et al. 2011; Wise 2016). In addition, multiple drug resistance (MDR) further limits the application of anticancer drugs in clinical practice (Dean, Fojo, and Bates 2005; Krishna and Mayer 2000; Liu 2009). To overcome these limitations, multiple technologies have been explored to enhance the selective targeting of drugs and to improve the pharmacological and therapeutic properties of drugs administered parenterally (Allen and Cullis 2014; Angeli et al. 2008). Among the development methods, nanotechnology-based drug delivery systems (DDSs) have been widely used as drug carriers to promote preferential accumulation in cancer cells (Farokhzad and Langer 2009; Mirjalili, Soltani, and Chen 2012; Ravichandran 2009). At present, several different types of nanoparticles (NPs), including polymeric NPs, polymeric micelles, dendrimers, liposomes, and nanocapsules, have been widely developed as vectors to assist the drugs in crossing biological barriers and specific barriers in tumors (Figueiredo et al. 2018; Fontana et al. 2017; Marrache, Pathak, and Dhar 2014).

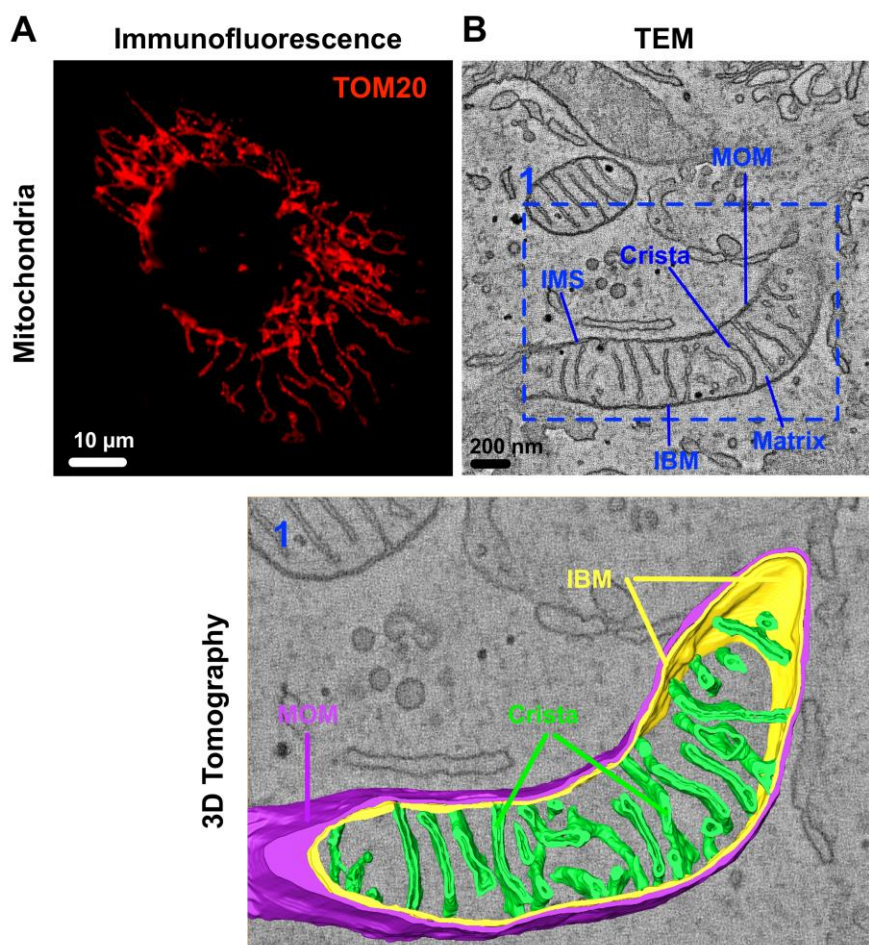
The poor selectivity of the genotoxic agent cisplatin greatly limits its clinical applications (Duan et al. 2016). Thus, the hydrophobic mitochondria-targeted cisplatin prodrug Platin-M was synthesized and subsequently loaded into mitochondria-targeted poly (lactic-co-glycolic acid)- $\beta$ -poly (ethylene glycol) nanoparticles (PLGA-PEG NPs) used for the treatment of neuroblastoma cells. The results showed PLGA-PEG NPs efficiently deliver Platin-M to accumulate inside mitochondria of neuroblastoma cells, profoundly boosting the anticancer activity compared with cisplatin (Marrache et al. 2014). The chemotherapy medication Doxorubicin (DOX) induces nuclei and mitochondria damage through inhibiting the progression of topoisomerase II and increasing ROS, exhibiting great anticancer activity (Granados-Principal et al. 2010; Yang et al. 2014). However, free DOX always enters the nuclei of tumor cells rapidly without tarrying in the mitochondria, weakening the efficiency of DOX. Through embedding DOX into hyaluronic acid (HA)-modified hydroxyapatite (HAP) nanoparticles (HAP-HA), the HAP-HA nanoparticles efficiently and simultaneously deliver the DOX into both mitochondria and nuclei of tumor cells. The DOX/HAP-HA nanoparticles not only enhance the anticancer efficiency of DOX, but also exhibit good stability and favorable biocompatibility (Xiong et al. 2016).

Altogether, an increasing number of studies demonstrate mitochondria to be a promising target for cancer therapy (Armstrong 2006; Schmitt and Zischka 2018, 2018; Weinberg and Chandel 2015).

Thus, a systematically understanding of the key pathophysiological differences between mitochondria in cancer cells and their counterparts in non-malignant cells will undoubtedly help us to develop novel drugs and to devise an optimal therapeutic strategy for cancer therapy in clinical practice.

#### 4. Mammalian mitochondrial ultrastructure

Mitochondria possess two structurally and functionally distinct membranes, the relatively smooth outer membrane and the highly folded inner membrane, which encompass two separate aqueous compartments, the intermembrane space (IMS) and the mitochondrial matrix (**Figure 1**). The mitochondrial outer membrane (MOM) forms the envelope of the organelles that separates the mitochondrial compartments from the cytosol, whereas the mitochondrial inner membrane (MIM) is subdivided into the inner boundary membrane (IBM) and tubular or lamellar crista membrane (CM). The IBM that is adjacent to the MOM harbors many protein translocases, and the CM comprises invaginations of the MIM towards the matrix that enhances its surface and is important for mitochondrial function. Between the MOM and MIM is a small hydrophilic layer named the intermembrane space. The IMS is the site for many exciting biological processes. The matrix is surrounded by the MIM and houses the mitochondrial genome (Brandt et al., 2017; Freya & Mannellab, 2000; Logan, 2006; van der Laan, Horvath, & Pfanner, 2016)





**Figure 1. Mitochondrial ultrastructure.** (A) Immunofluorescence image of mitochondria in U2OS cells. Mitochondria were visualized using antibody against the outer membrane protein TOM20. (B) The transmission electron microscopy (TEM) of mitochondria in U2OS cells. The lower panel is a magnification of electron tomographic reconstructions of mitochondria in blue area. The outer membrane (purple), inner boundary membrane (yellow), and crista (green) were superimposed on the tomographic slice and viewed from the top and side.

#### 4.1 Mitochondrial outer membrane

The MOM forms the border between mitochondria and the cytosol, controlling the exchange of material and signaling with other cellular compartments (Schorr and van der Laan 2018). Similar to the eukaryotic plasma membrane, the protein-to-phospholipid ratio of the MOM is around 1:1 by weight. The MOM contains large numbers of integral membrane proteins known as porins, e.g. the voltage-dependent anion channel (VDAC). These molecular channels and pores transport precursor proteins and small compounds across the outer membrane, which enables the communication between mitochondria and the cellular environment (Checchetto and Szabo 2018). The outer membrane also contains proteins that mediate several different cellular processes. The intrinsic apoptotic pathway is executed by mitochondrial outer membrane permeabilization (MOMP) under the control of proapoptotic BCL-2 family members (Martinou and Youle 2011).

Pten-induced kinase 1 (PINK1) protein is a mitochondrial localized serine/threonine kinase. In healthy mitochondria, PINK1 is constitutively imported into the matrix depending on the import receptor, translocase of the MOM subunit of 70 kDa (TOM70) and translocase of the MIM (TIM) (Greene et al. 2012; Kato et al. 2013). In the damaged or aged mitochondria, PINK1 accumulates on the outer membrane, activating the cytosolic Parkin's E3 ubiquitin ligase activity. Then, Parkin are recruited to dysfunctional mitochondria and ubiquitinate outer membrane proteins, inducing mitophagy, which ultimately leads to the elimination of defective mitochondria (Pickrell and Youle 2015).

Furthermore, mitochondrial outer membrane-bound dynamin-related proteins (DRPs), mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2), mediate the fusion of the MOM. The outer membrane also contains several adaptors of dynamin-related protein 1 (Drp1) that recruit Drp1 from the cytosol to the MOM. These proteins facilitate membrane remodeling and are involved in the fission of mitochondria through their ability to self-assemble and hydrolyze GTP (Friedman and Nunnari 2014). Some proteins on the MOM tether the organelle to other cellular membranes such as the ER. The mitochondria-ER contacts play important roles in lipid transport and calcium signaling (Klecker, Böckler, and Westermann 2014). All proteins on the MOM are synthesized on cytosolic ribosomes and contain internal targeting sequences. Distinct protein-targeting machineries mediate the insertion of these proteins into the mitochondrial outer membrane (Rapaport 2003).

#### 4.2 Mitochondrial intermembrane space

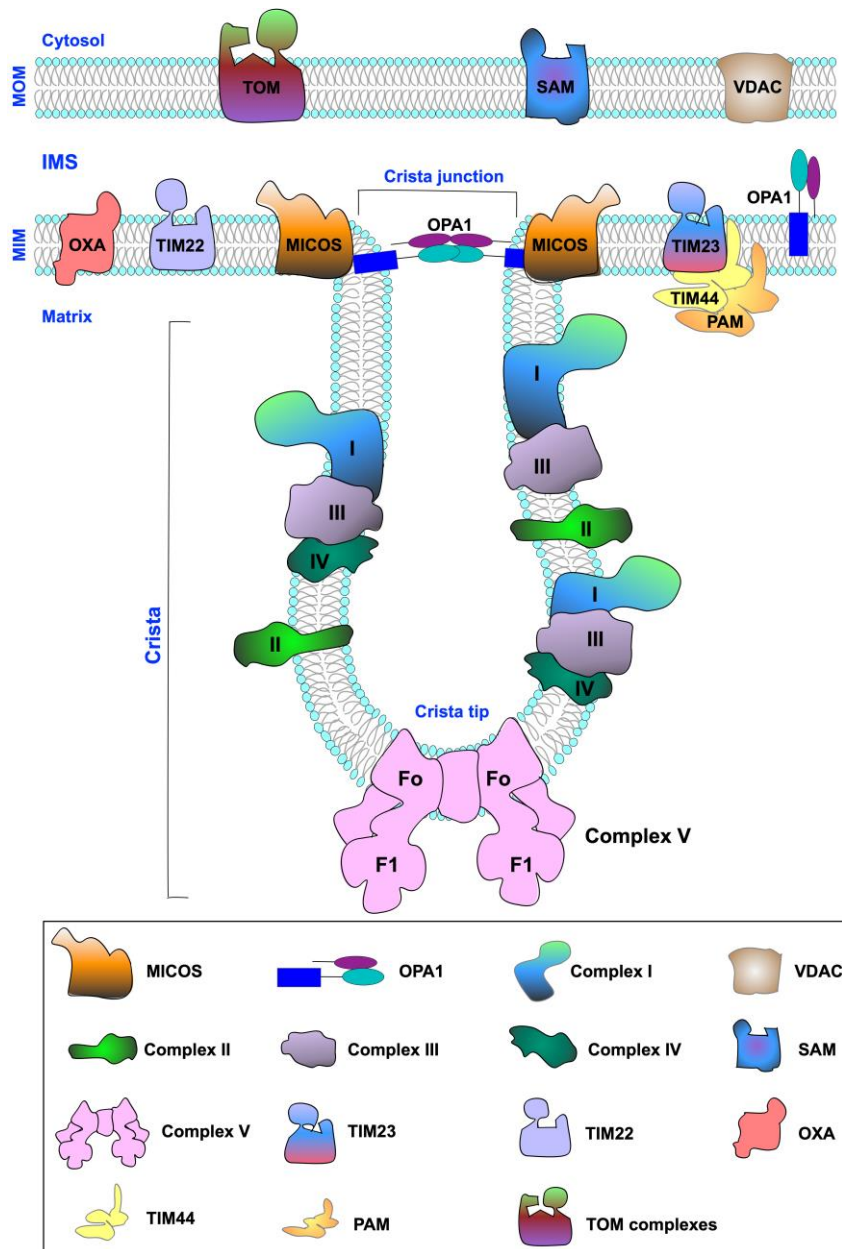
The mitochondrial intermembrane space is a small hydrophilic layer between the outer membrane and the inner membrane. Due to the free diffusion of small molecules, such as glutathione, between the cytosol and the IMS, it forms a reducing environment. Among ~1500 mitochondrial proteins, the proteins in IMS constitute about 10% of the mitochondrial proteome and play crucial roles in the communication with the cytosol and other mitochondrial compartments, in the uptake of metabolites, lipids, or metal ions, and in the regulation and execution of apoptosis (Backes and Herrmann 2018).

Most IMS proteins lack presequence and instead utilize the IMS localized oxidoreductase Mia40, which facilitates their translocation across the outer membrane in a reaction that is coupled to the formation of disulfide bonds within the protein. This process requires neither ATP nor the mitochondrial membrane potential ( $\Delta\Psi_m$ ) (MacPherson and Tokatlidis 2017).

#### **4.3 Mitochondrial inner membrane**

The mitochondrial inner membrane provides an extended, highly folded surface for selective transport and energy-coupling reactions. In addition, the highly folded inner membrane surface covers up to 50% of the total membrane area, which efficiently satisfies the accommodation of the oxidative phosphorylation subunits. Thus, the inner membrane is a protein-rich membrane with two domains differing in topology and protein composition (**Figure 2**) (van der Laan et al. 2016; Rampelt et al. 2017; Schwerzmann et al. 1986). The IBM is adjacent to the outer membrane and hosts most machinery for protein import to the inner mitochondrial compartments. The tubular or lamellar-like crista membranes are the deep invaginations of the inner membrane that provide an extended membrane surface for accumulation of the OXPHOS complexes (Complex I, II, III, IV, and F1F<sub>0</sub>-ATP synthase) (Gilkerson, Selker, and Capaldi 2003; Vogel et al. 2006; Zick, Rabl, and Reichert 2009). Through the electron microscopic studies, the OXPHOS complexes were estimated to represent 80% of the total intrinsic protein mass of the mitochondrial inner membranes (IBM and crista) (Baoukina, Marrink, and Tieleman 2012). The estimated protein/lipid ratio of the inner membrane is highly around 75:25 (D et al. 1990; Simbeni et al. 1991).

Crista membranes are connected to the IBM by narrow tubular or neck-like structures known as crista junctions (CJs) (Zick et al. 2009). The diameter of CJs is rather small, ranging from 12 to 40 nm (Nicastro et al. 2000; Perkins, Ellisman, and Fox 2003). The limited diameter maintains the asymmetric protein composition of the IBM and CM, and also efficiently acts as a dynamic diffusion barrier for movement proteins and metabolites between the intracristal space (ICS) and IMS and between the crista and IBM (Mannella et al. 1994; Rabl et al. 2009). The assembly and stabilization of the CJs structures mainly depend on the evolutionarily conserved mitochondrial contact site and cristae organizing system (MICOS) complex, a large and sophisticated inner membrane protein complex accumulated at CJs. The MICOS complex also establish contact sites between the inner and outer mitochondrial membranes by interacting with the outer membrane protein complexes (van der Laan et al. 2016; Pfanner et al. 2014; Wollweber, von der Malsburg, and van der Laan 2017).



**Figure 2. Ultrastructure and representative proteins of the mitochondrial inner membrane.** The inner membrane is sub-divided into the IBM and folded crista membranes. Protein translocases of the inner membrane 22 and 23 (TIM22 and TIM23) are preferentially located in the IBM, whereas OXPHOS complexes (complex I, II, III, IV) and the F<sub>1</sub>F<sub>0</sub>-ATP synthase (complex V) are enriched in the CM. The IBM and crista are connected by CJs, where the MICOS complex and OPA1 are enriched. The MICOS and F<sub>1</sub>F<sub>0</sub>-ATP synthase maintain the stabilization of CJs and crista, respectively. This picture is modified from van der Laan, Horvath, and Pfanner 2016.

#### 4.4 Matrix

The matrix is one of the most densely packed portions of the cell. Proteins in matrix constitute up to 50% of whole mitochondrial proteins (Goodsell 2010; Srere 1980). The matrix contains enzymes involved in such processes as the tricarboxylic acid cycle, oxidative phosphorylation, and beta oxidation of fatty acids. The entire set of mtDNA-encoded protein synthesis machinery is localized in the matrix, including mitochondrial DNA, polymerases, mitochondrial ribosomes, and transfer RNA (tRNA), among others. In addition, the folding and assembly of proteins derived from nuclear

and mitochondrial genomes are accomplished by the matrix-localized proteins, including the chaperonins heat shock protein 60 (HSP60) and HSP10, and chaperones including the HSP90 paralog tumor necrosis factor receptor-associated protein 1 (TRAP1) and the mitochondrial matrix 70 kDa heat shock protein (mtHSP70) (Kim et al. 2013; Xiwei Zheng, Cong Bi, Marissa Brooks 2015). Furthermore, the matrix acts as the main storage site for the  $\text{Ca}^{2+}$  pool (Vais et al. 2016).

## **5. Mitochondrial protein import pathways**

The mitochondrial proteome comprises approximately 1500 different proteins in mammals. Except for 13 core subunits of the respiratory chain encoded by mtDNA and synthesized in mitochondria, all other proteins are encoded by nuclear DNA and are synthesized as precursors in the cytosol (Hock and Kralli 2009). The unfolded precursors are subsequently imported into mitochondria via five major identified importing pathways depending on their mitochondrial targeting signals. Notably, each of the importing pathways is characterized by a different type of targeting signal (Schmidt, Pfanner, and Meisinger 2010; Wiedemann and Pfanner 2017).

The common entry gate for most precursors is the translocase of the outer membrane (TOM) complex. After passing through the TOM complex, the precursors use five different pathways to finish the localization of the mitochondrial subcompartments (Chacinska et al. 2009; Wiedemann and Pfanner 2017). In addition, a number of outer membrane proteins with  $\alpha$ -helical transmembrane segments localize to the mitochondrial outer membrane through the mitochondrial importing complex.

### **5.1 Presequence pathway to the matrix and mitochondrial inner membrane**

The presequence pathway is a classical route of protein import, which mediates the translocation of the vast majority of matrix proteins and many inner membrane proteins. Proteins imported via the presequence pathway are characterized by a cleavable sequence at the N-terminus called the mitochondrial targeting sequence (MTS). The presequence functions as targeting signals and usually consists of 10-80 amino acid residues that have the potential to form amphipathic helices with one hydrophobic and one positively charged face (Abe et al. 2000; Roise et al. 1986; Vögtle et al. 2009).

The cleavable presequence-carrying preproteins are transferred from the TOM complex to the TIM23 protein translocator complex. The TIM23 complex comprises ten subunits, Tim50, Tim23, Tim17, Tim44, Tim14 (Pam18), Tim16 (Pam16), Tim21, Pam17, mtHsp70 and Mge1 (Chacinska et al. 2005; Mokranjac and Neupert 2015). During the translocase in the TIM23 complex, the precursors with hydrophobic sorting signals are laterally released into the mitochondrial inner membrane. For the matrix localized protein, the TIM23 complex in cooperation with presequence translocase-associated motor (PAM) forms TIM23-PAM complex, which mediates the transportation of precursors into the matrix (Straub et al. 2016). Then the mitochondrial membrane potential drives the translocation of the presequence through the TIM23 complex and ATP powers the chaperone mtHSP70 of PAM (Horst et al. 1997; Kang et al. 1990). Lastly, the amino-terminal presequence of the precursor will be cleaved by the mitochondrial processing peptidase (MPP) (Hawlitsek et al. 1988).

### **5.2 Carrier pathway to the mitochondrial inner membrane**

Different from the precursors with an N-terminal cleavable presequence, the precursors translocated through the carrier pathway contain three to six uncleavable discontinuous internal targeting signals

(Chacinska et al. 2009; Saitoh et al. 2007; Wiedemann and Pfanner 2017). Because the precursors usually contain a multispinning hydrophobic structure, cytosolic localized chaperones (such as HSP70) often bind precursors to prevent their aggregation (Li et al. 2009; Young, Hoogenraad, and Hartl 2003; Zara et al. 2009). The formed complexes of the precursors and chaperones are then recruited to the receptor of mitochondrial outer membrane TOM70 which possesses binding sites for the precursors and chaperones (Li et al. 2009; Young et al. 2003). The interaction of TOM70 with the precursors further mediates the translocation of the precursors across the TOM complex, usually consisting of TOM40 (Wiedemann, Pfanner, and Ryan 2001). After being translocated through TOM40, the precursors are transferred by the TIM9-TIM10 chaperone through the intermembrane space (Curran et al. 2002; Webb et al. 2006). Then the precursor translocase of the inner membrane, the TIM22 complex, drives the membrane insertion of imported proteins in a  $\Delta\Psi_m$  dependent reaction (Lithgow and Schneider 2010; Wagner et al. 2008).

### **5.3 Oxidative folding pathway of the intermembrane space**

Most intermembrane space proteins are small proteins that are synthesized without cleavable presequence but contain Cys motifs (Gabriel et al. 2007). The precursors are imported by the TOM complex and the mitochondrial intermembrane space import and assembly (MIA) proteins (Chacinska et al. 2004a; Naoé et al. 2004). MIA includes the protein disulfide carrier Mia40 and the sulfhydryl oxidase Erv1 (essential for respiration and viability 1) (Allen et al. 2005; Chacinska et al. 2004b; Terziyska et al. 2005). Erv1 oxidizes Mia40 by transferring a disulfide bond (S-S) to Mia40 (Bien et al. 2010; Bihlmaier et al. 2007; Stojanovski et al. 2008). After translocation across the TOM complex, the precursor proteins bind to the oxidized Mia40 via forming a transient disulfide bond between the targeting signal of the precursor and Mia40 (Allen et al. 2005; Bihlmaier et al. 2007). Then, Mia40 transfers the disulfide bond to the precursor. Thus, Mia40 returns to the reduced form and is recycled for further re-oxidized by Erv1. The oxidized precursor proteins are released to the mitochondrial intramembrane space (Stojanovski et al. 2008).

### **5.4 $\beta$ -barrel pathway of the mitochondrial outer membrane**

The mitochondrial outer membrane localized proteins contain two different types of integral proteins,  $\alpha$ -helical proteins and  $\beta$ -barrel proteins (Schmidt et al. 2010; Wiedemann and Pfanner 2017). The two kinds of precursor proteins are sorted to the mitochondrial outer membrane through two different pathways (Wiedemann et al. 2003; Wiedemann and Pfanner 2017). Similar to the above protein import pathway, the  $\beta$ -barrel proteins are translocated to the mitochondrial outer membrane through the TOM complex (Ahting et al. 1999; Model et al. 2001; Paschen et al. 2003). Simply, the precursors of  $\beta$ -barrel proteins are initially imported by TOM complex. Then the transported precursors bind to small TIM chaperones existing in the intermembrane space. Lastly, the sorting and assembly machinery (SAM) facilitates the insertion of the precursor into the outer membrane (Chan and Lithgow 2008; Kozjak et al. 2003; Pfanner et al. 2004). The receptor TOM22 and the peripheral membrane protein Sam37 link TOM and SAM into a transient supercomplex that promotes the efficient transfer of precursor proteins (Meisinger et al. 2004; Wideman et al. 2010; Yamano, Tanaka-Yamano, and Endo 2010). Folding of the  $\beta$ -barrel occurs at Sam50-Sam35, followed by release of the  $\beta$ -barrel into the lipid phase of the MOM (Wideman et al. 2010).

### 5.5 $\alpha$ -helical transmembrane proteins of the mitochondrial outer membrane

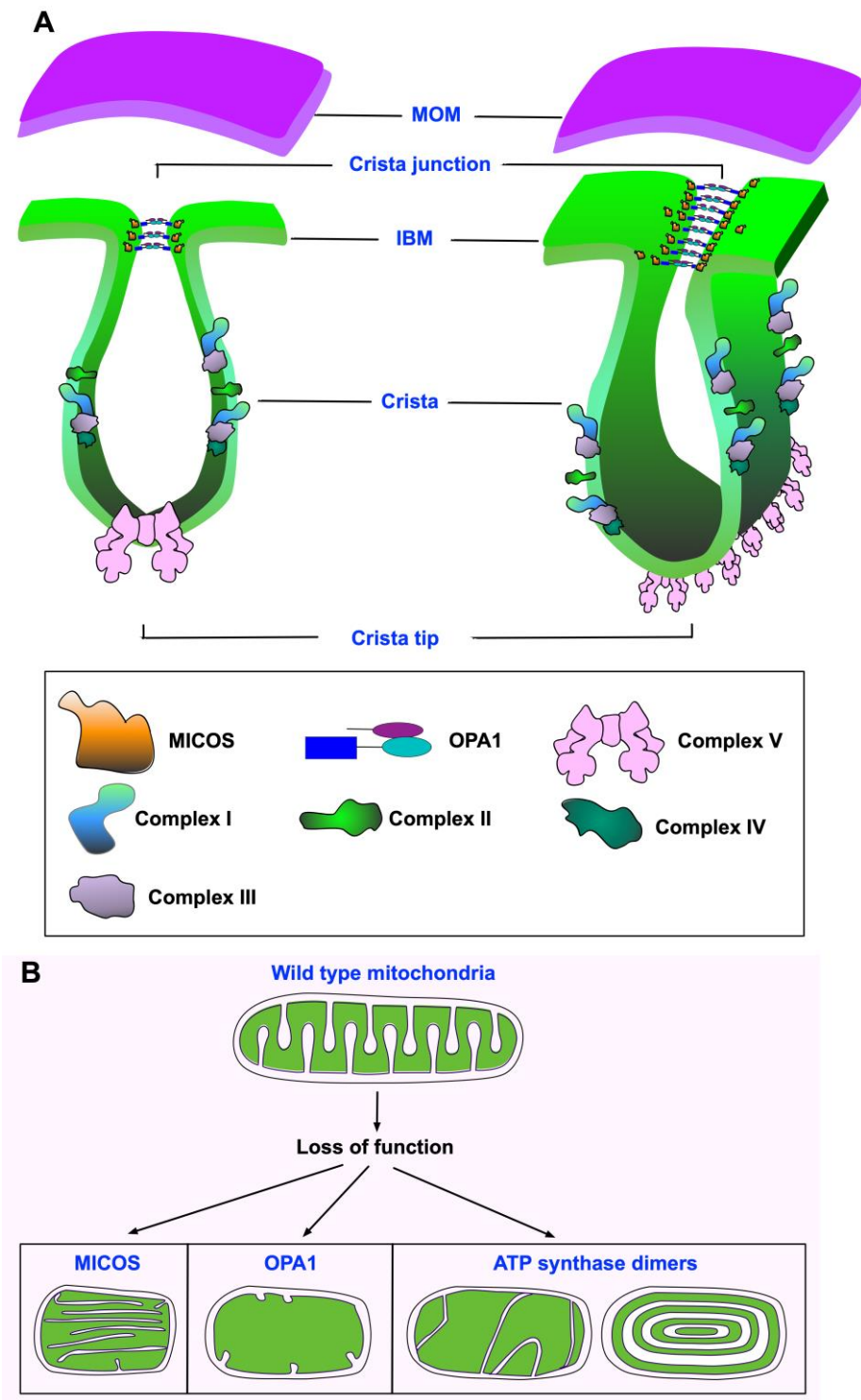
The biogenesis of  $\alpha$ -helical outer membrane proteins is less understood than the  $\beta$ -barrel proteins at present (Dukanovic and Rapaport 2011; Ellenrieder, Mårtensson, and Becker 2015). Three major protein classes are involved: signal-anchored proteins, tail-anchored proteins, and polytopic (multispanning) outer membrane proteins (Wiedemann and Pfanner 2017). Different import pathways mediate the sorting of these three integral outer membrane protein classes. For the part of polytopic MOM precursors, the proteins are recognized by TOM70 and are subsequently transferred to the insertase of the mitochondrial outer membrane, also known as MIM complex. The MIM complex promotes the efficient insertion of the polytopic proteins (Becker et al. 2011; Papić et al. 2011). The other polytopic proteins as well as tail-anchored MOM proteins may be inserted into the outer membrane in a lipid-assisted manner, possibly independent of specific proteinaceous machinery (Kemper et al. 2008; Krumpe et al. 2012; Setoguchi, Otera, and Mihara 2006). Except for the polytopic proteins, the precursors of proteins with an N-terminal signal anchor sequence are also dependent on the MIM complex for insertion into the mitochondrial outer membrane (Wiedemann and Pfanner 2017).

## 6. Formation and maintenance of crista

The mitochondrial inner membrane is highly folded, having a four-fold greater surface area than the outer membrane (Frey and Mannella 2000; Mannella 2006). Depending on the degree of membrane curvature, the inner membrane is subdivided into different morphologically distinct domains: the unfolded or flat IBM, the curved tubular or flat lamellar crista membranes, and the highly curved crista junction and crista tip with negative membrane curvature and positive membrane curvature, respectively (**Figure 3A**) (van der Laan et al. 2016). The crista structure can vary in length, width, lateral alignment, rigidity, and angularity (Mannella 2006; Zick et al. 2009). The inner boundary membrane is enriched for the import and assembly machinery for the mitochondrial proteome (Vogel et al. 2006). The crista membrane houses assembled electron transport chain protein complexes and ATP synthases, which efficiently couple the processes of oxidative phosphorylation and ATP generation. The narrow crista junction area helps to restrict proteins to a particular area of the inner membrane, e.g. the proapoptotic factor cytochrome c is limited to the ICS to avoid activation of the apoptotic process (Friedman et al. 2015; Olichon et al. 2003). The dimerization of adenosine triphosphatases (ATPases) at the crista tip stabilizes crista membrane rims (Acehan et al. 2011; Davies et al. 2012).

The morphology of crista varies in size and shape, appearing as flat lamellae, extended tubules or heterogeneous membrane sacs (Frey, Renken, and Perkins 2002; Mannella et al. 2002; Perkins et al. 1997; Zick et al. 2009). Depending on the environmental state, the crista membranes usually undergo extensive changes such as increasing the abundance of the crista membrane, tightening between crista membranes, and opening of crista junction (Hackenbrock 1966). The mechanisms by which inner membrane domains are established and maintained are poorly understood at present, but several protein complexes and phospholipids have been found to mediate the biogenesis and maintenance of the sophisticated crista structure. The aberrant expression of three main protein complexes, optic atrophy 1 (OPA1), MICOS and ATP synthases, leads to aberrant crista morphology, suggesting pivotal roles in crista architecture (**Figure 3B**). Furthermore, the composition of the mitochondrial

lipid or the level of scaffolding proteins such as prohibitin are proposed to determine the inner membrane structure (Del Dotto et al. 2018; Ikon and Ryan 2017; Kozjak-Pavlovic 2017; Quintana-cabrera et al. 2018; Zerbes et al. 2012; Zick, Rabl, and Reichert 2009).



**Figure 3. Protein complexes involved in characteristic morphology of crista.** (A) Schematic representation with the main regulators of crista morphology positioned in crista. (B) Representative phenotypes resulting from the ablation of key regulators of crista structure. This picture is modified from Del Dotto et al. 2018.

## 6.1 OPA1

OPA1 is a dynamin-related guanosine triphosphatase (GTPase) located in the inner membrane facing the intermembrane space. It governs the delicate balance between fusion and fission in the dynamic mitochondrial network. In addition, OPA1 is capable of forming oligomers or higher molecular weight complexes that are involved in crista structure organization (MacVicar and Langer 2016; Pernas and Scorrano 2016). Human *OPA1* gene consists of 31 exons and results in synthesis of at least eight different messenger RNA (mRNA). The eight OPA1 transcripts encode proteins of 924-1014 amino acids (Delettre et al. 2001). After being imported into mitochondria, OPA1 precursor proteins are processed by the MPP, producing the mitochondrial inner membrane-bound long form of OPA1 (L-OPA1). In humans, four L-OPA1 polypeptides contain both S1 and S2 proteolytic cleavage sites, whereas the other four polypeptides contain the S1 proteolytic site alone. The L-OPA1 polypeptides are further processed by the inner membrane peptidases OMA1 and YME1L at the S1 and S2 site, respectively. Proteolysis at these two sites results in loss of the transmembrane domain of L-OPA1, hence generating a soluble short form of OPA1 (S-OPA1).

In healthy conditions, due to the limited OMA1 activity, mature OPA1 undergoes constitutive processing at S1 and S2, leading to the accumulation of noncleaved, long and short form of OPA1. The L-OPA1 and S-OPA1 assemble into oligomeric complexes and situates along the crista structure. The oligomeric complexes are indispensable for inter-crista membrane tethering and play key roles in maintaining crista ultrastructure (Frezza et al. 2006; Yamaguchi et al. 2008). In stress conditions or apoptotic stimulation, OMA1 is activated and causes the reduction of L-OPA1 and the increase of S-OPA1. The disturbance of the L-OPA1 and S-OPA1 balance further induces abnormal crista ultrastructure (MacVicar and Langer 2016). Although S-OPA1 does not appear to play a key role in steady-state maintenance of crista morphology, increasing evidences suggests that it may play a role in crista stapling by increasing OPA1 oligomerization between apposing crista membranes (MacVicar and Langer 2016; Pellegrini and Scorrano 2007).

## 6.2 MICOS complex

The mitochondrial contact site and cristae organizing system also known as the MICOS complex are a large inner membrane complex of proteins localized in the crista junction that function in organizing the inner membrane into folds, which is crucial for determining crista structure and formation of crista junction (Davies et al. 2012; Muñoz-Gómez et al. 2015). Compared with the six identified subunits of the MICOS complex in yeast, the diversity of subunits and interactions seems much greater in the mammalian system. Eight subunits of MICOS (MIC60, MIC10, MIC19, MIC25, MIC23, MIC27, MIC13, and MIC14) and one putative interactor (DnaJC11) have thus far been identified as components of the MICOS complex, with possibly more to come (Alkhaja et al. 2012; An, Shi, He, Lui, Liu, Huang, and M Saeed Sheikh 2012; Darshi et al. 2011; Genin et al. 2016; Ioakeimidis et al. 2014; Koob et al. 2015; Weber et al. 2013). Except for the two peripheral inner membrane subunits MIC25 and MIC14, the other subunits are embedded into the inner membrane with domains facing the intermembrane space. The depletion of a single subunit, especially the core components of the MICOS complex, MIC60 and MIC10, will lead to a reduction in the number of crista junction and a defect in lamellar crista shape (Harner et al. 2011; Hoppins et al. 2011; Rabl et al. 2009).

The MICOS complex interacts with the outer membrane SAM complex. Together with the outer



membrane components metaxin1-3 and the full-length DnaJC11, the MICOS complex further forms a high molecular super-complex called the mitochondrial intermembrane space bridge (MIB) complex. Through the interaction of core subunit MIC60 with TOM, SAM and fusion protein Ugo1, and outer membrane protein porin (also known as VADC), the MIB complex efficiently couples the crista organization system with the mitochondrial import machineries (Harner et al. 2011; von der Malsburg et al. 2011; Xie et al. 2007).

### **6.3 Dimeric ATP synthase**

The F<sub>1</sub>F<sub>0</sub>-ATP synthase (ATP synthase) is a highly conserved rotary motor enzyme accomplishing the energy conversion in mitochondria. It is a multi-subunit assembly complex and consists of two structure domains, a matrix localized globular domain (F<sub>1</sub>) and an intrinsic membrane domain (F<sub>0</sub>), linked together by a central stalk and a peripheral stalk (Jan Pieter Abrahams, Andrew G. W. Leslie 1994; Walker and Dickson 2006). The process of ATP generation catalyzed by ATP synthase can be divided into three steps: (1) the inward passage of proton via F<sub>0</sub> domain converts electrochemical proton gradient energy across the mitochondrial inner membrane into subunit rotation; (2) the rotating transmission device, the central stalk, rotates in a clockwise manner and transmits the energy to the catalytic sites of F<sub>1</sub> domain; and (3) the catalytic sites convert mechanical energy of rotation into the chemical bond between the ADP-O and Pi, which synthesizes ATP (Jan Pieter Abrahams, Andrew G. W. Leslie 1994; Walker and Dickson 2006).

Except for ATP generation, ATP synthase is observed to form dimers in rows along the highly curved crista ridge, which is involved in the biogenesis of the crista membrane (Davies et al. 2012; Gavin 2004; Minauro-Sanmiguel, Wilkens, and Garcia 2005; Strauss et al. 2008). Electron cryotomography (cryoET) revealed that the mammalian ATP synthase is arranged in long ~1 µm rows of dimeric super-complexes, located at the tip of crista membranes. The interface of the dimer is formed by contacts on both F<sub>1</sub> and F<sub>0</sub> domains and the dimer ribbons enforce a strong local curvature on the crista membrane with a 17 nm outer radius (Strauss et al. 2008). The dimers form a V-shaped structure with an angle of 86° between monomers (Hahn et al. 2016). The two F<sub>1</sub> subcomplexes within each dimer are consistently 28 nm apart, while the distance between adjacent dimers along the rows is variable among the different species (Davies et al. 2012). Deletion of the two accessory subunits that mediate the dimer formation leads to the loss of ATPase dimerization, consequently altering the crista architecture (Arselin et al., 2003).

### **6.4 BAR domain protein**

The Bin-Amphiphysin-Rvs (BAR) domain family of proteins are central regulators of diverse cellular processes in all eukaryotes, characterized by their modular architecture. BAR domain proteins sense and interact with membranes via non-specific electrostatic interactions, sculpting phosphoinositide-rich membranes and generating membrane curvature, invaginations (e.g. endocytosis), and protrusion (e.g. filopodia). According to their preferences for membranes of different curvatures, BAR domain proteins are classified into three major subfamilies: the classical crescent-shaped N-BAR (Peter et al. 2004; Tarricone et al. 2001), the more extended and less curved *Fes/CIP4* homology BAR (F-BAR) (Frost, De Camilli, and Unger 2007; Henne et al. 2007; Itoh et al. 2005), and the inverse curvature I-BAR (Lee et al. 2007; Millard et al. 2005).

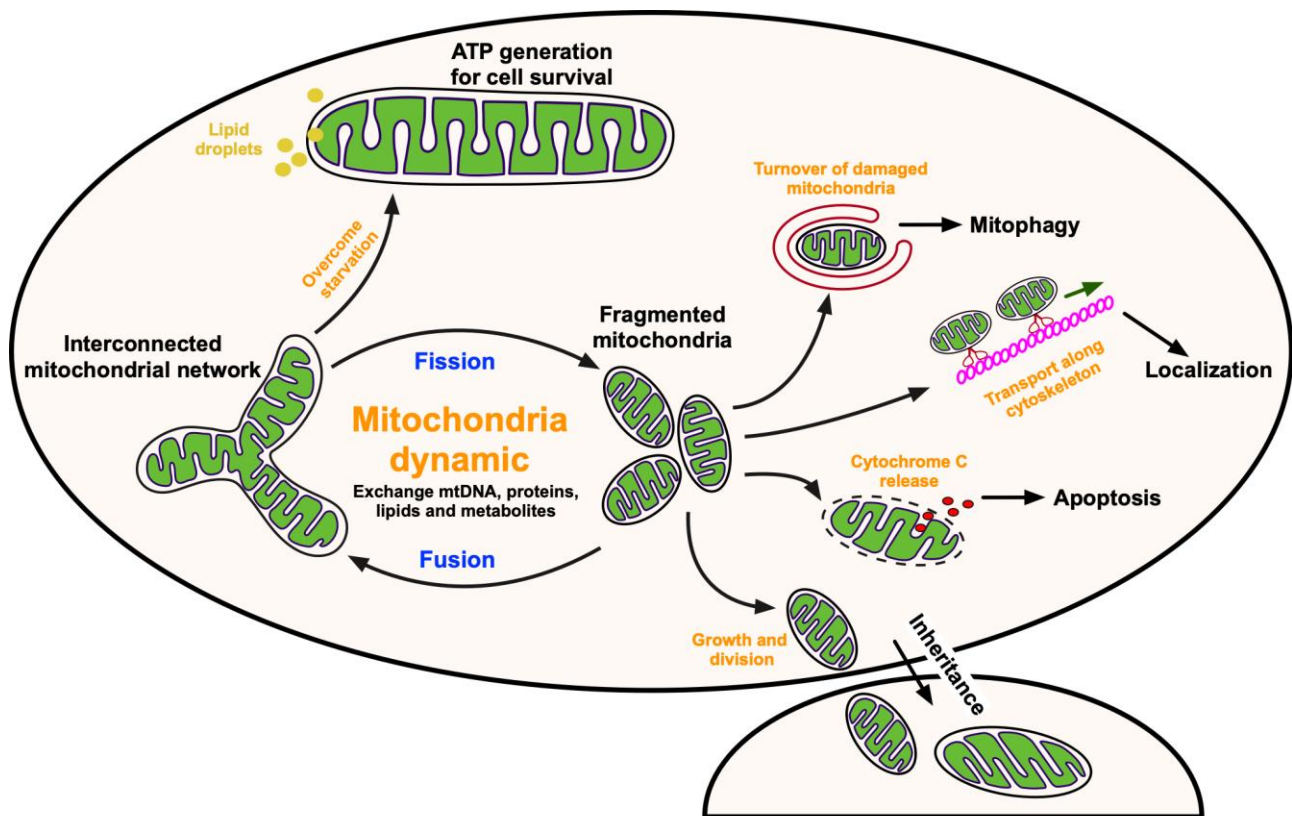
Most of identified N-BAR domain proteins contain an N-terminal amphipathic helix and prefer membranes of high positive curvature. F-BAR domain protein has a strong preference for positively curved membranes (Carman and Dominguez 2018; Saarikangas, Zhao, and Lappalainen 2010). In contrast to N-BAR and F-BAR domain proteins which induce the formation of membrane invagination, I-BAR domain proteins prefer to sense negatively curved membranes and help maintain membrane protrusion (Mattila et al. 2007). Except for BAR domain, most BAR domain proteins contain additional domains that often function together with the BAR domain in membrane binding and in protein-protein interactions. For example, the N-BAR domain protein endophilin contains one SH3 domain at the C-terminus, mediating the binding to the interaction proteins (Ringstad, Nemoto, and De Camilli 2002; Verstreken et al. 2003).

At the structural level, the BAR domains are dimers of an antiparallel helix bundle that display varying degrees of intrinsic curvature (Peter et al. 2004; Shimada et al. 2007; Weissenhorn 2005). Furthermore, the BAR domains can oligomerize into helical scaffolds to promote membrane deformation (Carman and Dominguez 2018; Mim et al. 2012). *In vitro* studies, the purified BAR domain proteins were verified to sculpt membrane-like liposome into tubes and vesicles (Frost et al. 2008; Peter et al. 2004). Furthermore, cryoET reconstruction has shown how the F-BAR domain protein generates membrane tubulation and visiculation (Frost et al. 2008). These studies suggest the essential roles of the BAR domain in dynamic membrane remodeling and in intracellular vesicle budding.

Except for endocytosis, apoptosis, and cell-cell fusion, membrane sculpting is also required for crista membrane biogenesis and maintenance. At present, several proteins have also been verified to control crista morphology in addition to the above-introduced proteins (An, Shi, He, Lui, Liu, Huang, and M. Saeed Sheikh 2012; Guarani et al. 2015; John et al. 2005). So far, the BAR domain superfamily of proteins has mainly been identified to function in the endocytic pathway; only one N-BAR domain protein FAM92A1 has been found to play a key role in maintenance of mitochondrial membrane structure, especially mitochondrial inner membrane architecture (Wang et al. 2019).

## **7. Mitochondrial dynamics**

Mitochondria are highly dynamic organelle constantly undergoing fission and fusion, referred to as ‘mitochondrial dynamics’. Through coordinate cycles of fusion and fission, mitochondria maintain their shape, distribution, and size (Tilokani et al. 2018; Westermann 2010). These processes are crucial for mitochondrial inheritance and for many cellular processes such as cell cycle, immunity, apoptosis and mitochondrial quality control. Furthermore, the frequently and rapidly dynamic process also allows for a transfer of information via exchange of mtDNA, proteins, lipids, and metabolites (Balaban, Nemoto, and Finkel 2005; Chen, Chomyn, and Chan 2005; Chen, McCaffery, and Chan 2007; Frank et al. 2001; Ishihara et al. 2009; Tilokani et al. 2018; Westermann 2010) (**Figure 4**).



**Figure 4. Physiological roles of mitochondrial dynamics.** The biological function of mitochondrial fusion and fission. Fusion process is important for the dissipation of metabolic energy and defense of aging. Fission is required for inheritance and partitioning of mitochondria during cell division. Mitochondria division process also participates in intrinsic apoptosis through the release of cytochrome c from intracristal space to cytosol. Furthermore, mitochondria with low membrane potential or reduced OPA1 level after fission are prone to removal by the selectively mitochondrial autophagy, mitophagy. This picture is modified from Westermann 2010.

The core machinery of dynamic transitions is mainly executed by highly conserved large GTPases belonging to the Dynamin family, with Drp1 and mitofusins (Mfn1/2) separately controlling fission and fusion of the MOM. In addition to the fusion of the MOM, another GTPase, OPA1, mediates the fusion of the mitochondrial inner membrane (Chan 2006a; Chang and Blackstone 2010; Suen, Norris, and Youle 2008).

### 7.1 Mitochondrial fission

Mitochondrial fission requires the coordination of cytoplasmic, cytoskeleton and organellar elements. The fission process consists of three key steps: (1) the ER marks the mitochondrial pre-constricted fission site on the mitochondria after undergoing stimulation of fission signals; (2) the cytosolic localized inactive form of Drp1 is recruited around the marked fission sites and assembled into a spiral-shaped superstructure; (3) the higher-order assembled Drp1 leads to membrane constriction and induces the recruitment of Dynamin 2 (Dyn2) to accomplish the membrane scission through hydrolyzing GTP (**Figure 5A**) (Loson et al. 2013; Otera, Ishihara, and Mihara 2013; Tilokani et al. 2018).

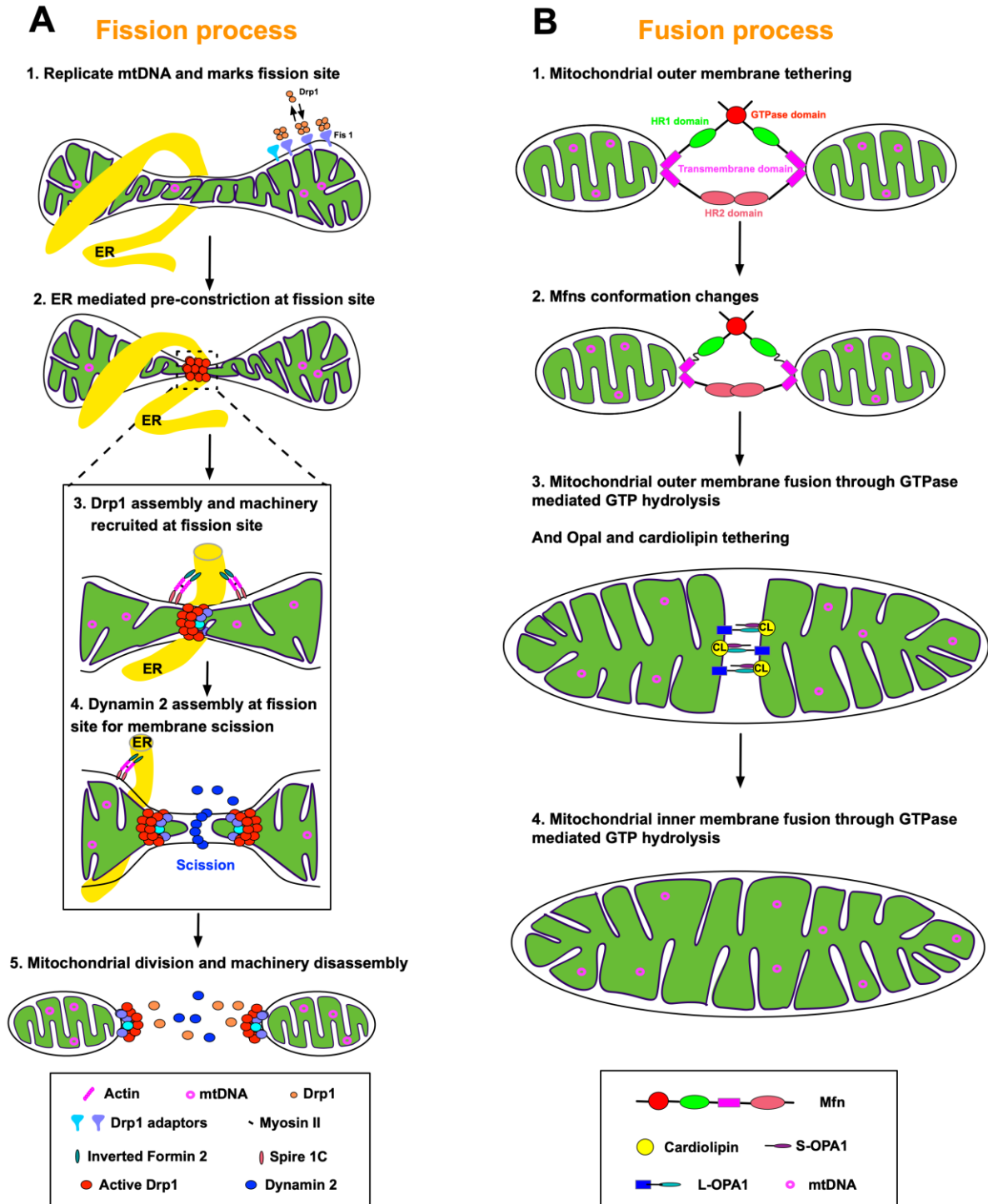
The mitochondrial pre-constricted fission sites usually occur at the contact sites between mitochondria and ER (Friedman et al. 2011). The ER-localized inverted formin 2 (INF2) and mitochondrial anchored formin-binding Spire 1C induce actin polymerization through the recruitment of myosin II at the ER-mitochondrial interface. The recruitment of myosin II provides the mechanical force for the actin cable, which drives the constriction of the mitochondrial membrane. With the decrease of mitochondrial diameter, the cytosolic Drp1 is recruited to the mitochondrial outer membrane via its adaptors.

In mammals, four integral mitochondrial outer membrane adaptors are responsible for the recruitment of Drp1: mitochondrial fission protein 1 (Fis1), mitochondrial fission factor (Mff), and mitochondrial dynamics proteins of 49 and 51 kDa (MiD49 and MiD51) (Loson et al. 2013). The recruited Drp1 proteins are subsequently oligomerized into a ring-like structure around the pre-constriction site. The GTP hydrolysis depending on GTPase activity of Drp1 leads to a conformational change, enhancing pre-existing mitochondrial constriction (Pernas and Scorrano 2016).

Although Drp1 has roles in mitochondrial membrane constriction, Drp1 is unable to terminate fission alone. A recent study showed that another GTPase protein Dyn2 is recruited to the Drp1-mediated mitochondrial constriction neck. The assembled Dyn2 terminates membrane scission, leading to the formation of two daughter mitochondria (Lee et al. 2016).

## **7.2 Mitochondrial fusion**

Fusion is an adaptation to intracellular life, facilitating communication between the mitochondria and host cell. Unlike the fission process, the fusion of mitochondrial outer and inner membrane are executed by different proteins (Chan 2006b; Lee and Yoon 2016). Globally, it is characterized by four different steps: (1) The two closely contacted mitochondria are tethered together through the insertion of dynamin-related MOM proteins Mfn1 and Mfn2 into the apposing MOM; (2) The dimeric conformational change of mitofusin shortens the distance between two mitochondria, leading to an increase in the contact surface of two membranes (Brandt et al. 2016; Ishihara 2004; Koltzsch et al. 2003); (3) GTPase-dependent power stroke or GTP-dependent oligomerization mediates the fusion of the outer membrane. Following the MOM fusion, the L-OPA1 interacts with cardiolipin (CL), forming complexes, and the S-OPA1 stimulates GTPase activity or triggers structural rearrangements, promoting the fusion of mitochondrial inner membrane; (4) The oligomerized OPA1 coordinates with Mfn1 driving fusion of the inner membrane depending on GTP hydrolysis (**Figure 5B**) (Cipolat et al. 2004; Mishra et al. 2014).



**Figure 5. A schematic of mitochondrial fission and fusion.** (A) Representative process of fission. Briefly, mark the mitochondrial pre-constriction site by ER, recruit the cytosolic localized Drp1 to the marked site, and assemble into a ring-like structure, mediating the pre-constriction of the membrane. Then, the polymerization of Myosin IIa at the mitochondrial-ER contact site provides the mechanical force for Drp1-mediated constriction. Lastly, Dyn2 is recruited to the constriction neck where it assembles and terminates membrane scission leading to two daughter mitochondria. (B) Representative process of fusion. The mitofusions tether the outer membrane of two opposing mitochondria, where the conformational change mediated by GTP hydrolysis increases the contact site. Then, GTP-dependent oligomerization induces the fusion of the outer membrane. Following the MOM fusion, OPA1 and cardiolipin drive fusion of the inner membrane. This picture is modified from Tilokani et al. 2018.

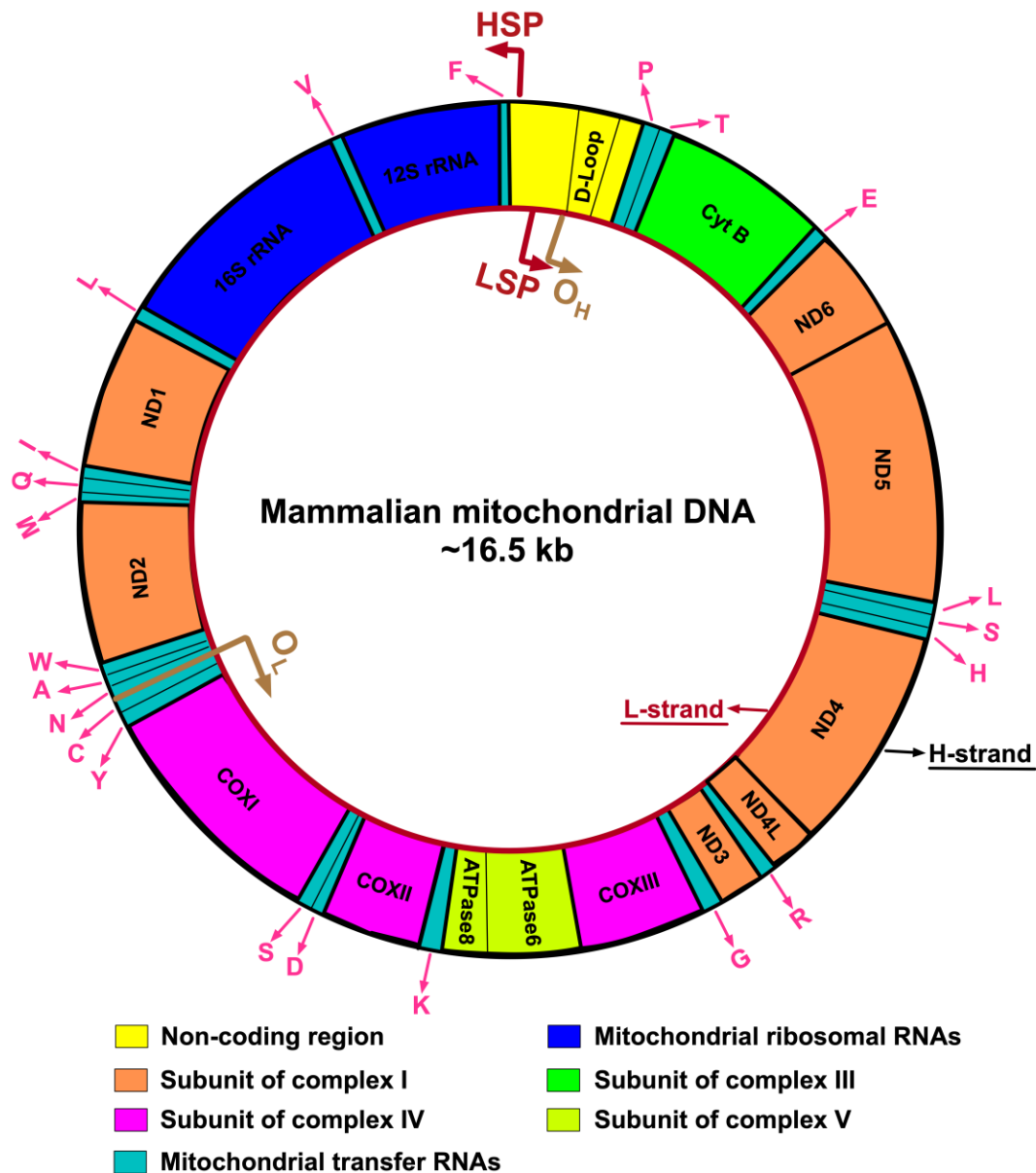
## 8. Mitochondrial DNA

In mammalian cells, approximately 3-5% of total proteins are mitochondrial localized proteins. The vast majority are encoded by the nucleus genome, translated by the cytosolic ribosomes, and transported into mitochondria mediated by different importing mechanisms depending on the sub-mitochondrial localization (Hoogenraad and Ryan 2007). Only 13 proteins, the necessary subunits of the OXPHOS complexes, are encoded by mitochondrial DNA and translated by mitochondrial ribosomes (mitoribosomes).

### 8.1 mtDNA structure

Mitochondrial DNA of mammals is a closed circular double-strand molecule of approximately 16.5 kb that encodes for 37 genes (Shoubridge & Wai, 2007; Spelbrink, 2010). Except for the necessary subunits of OXPHOS complexes, mtDNA also encodes all of the 24 RNA components, including 22 tRNA and 2 ribosomal RNA (rRNA) required for mitochondrial-encoded protein synthesis (**Figure 6**). (Anderson, Bankier, Barrell, de Bruijn, et al., 1981; Clayton, 2000; Park & Larsson, 2011). Due to a bias distribution of guanines between the two helical strands, the two strands of mtDNA are separated into a heavy strand (H-strand, black color in **Figure 6**) and a light strand (L-strand, red color in **Figure 6**) based on density difference in alkaline CsCl<sub>2</sub> gradients (Clayton 2003). The coding genes distribute unevenly between H-strand and L-strand. The transcripts of L-strand contain 8 tRNAs (P, E, S, Y, C, N, A, Q) and 1 mRNA of ND6. While the transcripts of H-strand are more numerous than those of the transcript of L-strand, including 2 rRNAs (12S and 16S), 14 tRNAs (F, V, L, I, M, W, D, K, G, R, H, S, L, T) and 10 mRNAs of polypeptides (ND1, ND2, ND3, ND4, ND4L, ND5, CYTB, COI, COII, COIII, ATP6 and ATP8) (Clayton, 2000; Taanman, 1999).

Furthermore, the mtDNA contains one hallmark non-coding region with a size of 1124 bp called the D-loop region. It contains essential replication and transcription elements, thus acting as a promoter for both H-strand and L-strand at the initial step of the transcription process. The D-loop region is a hot spot for mtDNA alterations, and it contains two hypervariable regions (HV1 at positions 16024-16383 and HV2 at positions 57-372) (Clayton 2000). The mutation of this region serves as a potential sensor for cellular DNA damage and a marker for disease development (Cai et al. 2011; Lee et al. 2004). Furthermore, the mutations of several regions of mtDNA D-loop region have been identified in breast cancer tissue (Richard et al. 2000; Tan, Bai, and Wong 2002).



**Figure 6. Schematic structure of human mtDNA.** The genomic organization and structural features of human mtDNA are depicted in a circular genomic map. The D-loop regulatory region is expanded and shown above. The 13 mtDNA-encoded protein regions are marked in protein abbreviations. The tRNA genes are denoted by the single-letter amino acid code. The light-strand promotor and heavy-strand promotor are shown with LSP and HSP, respectively. The origin of H-strand replication ( $O_H$ ) and L-strand replication ( $O_L$ ) are also indicated in brown. This picture is modified from Kelly and Scarpulla et al. 2004.

## 8.2 Replication of mtDNA

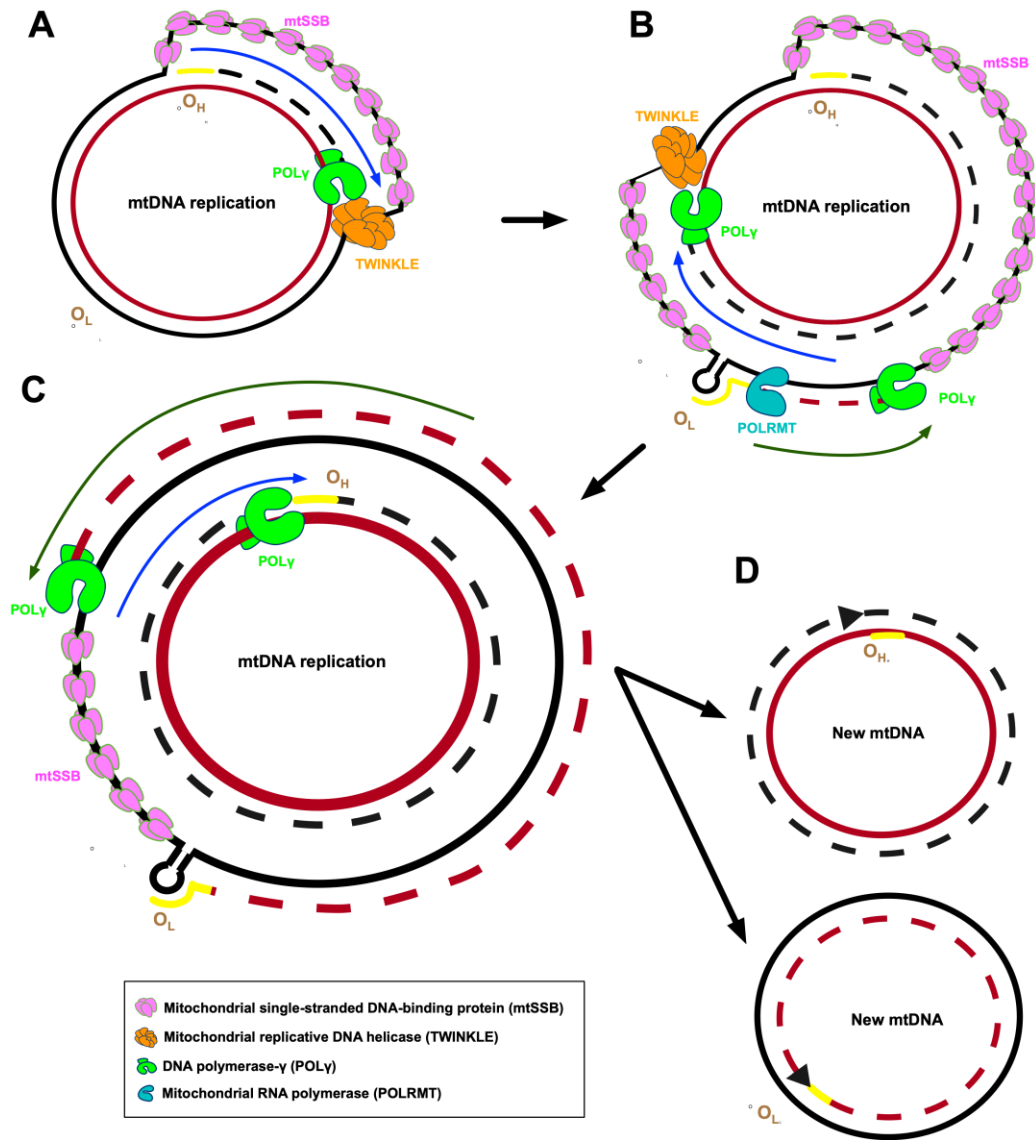
Over the past several decades, numerous efforts have been made to examine the model of mtDNA replication and transcription. So far, among the proposed mtDNA replication mechanisms, there are two main patterns, strand-displacement replication model (Clayton 1982) and strand-coupled replication model (Holt, Lorimer, and Jacobs 2000; Yang et al. 2002). In the study of Holt et al., the authors found the replication intermediates coexist in both human and mouse mitochondria (Holt et al. 2000). In the strand-coupled replication model, closed circular mtDNA begins leading-strand synthesis for the synthesis of lagging-strand early in replication. The synthesized lagging-strand

replication initiates at multiple sites, probably involving discontinuous synthesis of multiple short-fragments ('Okazaki').

Another asymmetric, the strand-displacement model (SDM) is another model of mtDNA replication (Clayton 1991). Different from the strand-coupled replication model, the mtDNA synthesis occurs continuously on both strands basing on the SDM. Therefore, in order to ensure coordination of DNA synthesis, mtDNA contains a dedicated origin of DNA replication on each strand, the heavy-strand origin ( $O_H$ ) and the light-strand origin ( $O_L$ ) (**Figure 6**). Many replication factors are involved in the replication process. The core replicative polymerase- $\gamma$  ( $POL\gamma$ ) is the only DNA polymerase executing mtDNA replication (Fan et al. 2006; Gray and Tai Wai Wong 1992). Because  $POL\gamma$  cannot directly use double-strand DNA as a template on its own, mitochondrial single-stranded DNA (ssDNA)-binding protein (mtSSB) stimulates DNA helicase TWINKLE to unwind the mtDNA duplex by working together with  $POL\gamma$  at the replication fork (Korhonen et al. 2004). The produced long stretches of ssDNA are stabilized by tetramerization of mtSSB (Kaguni 2004; Korhonen, Gaspari, and Falkenberg 2003). Furthermore, mitochondrial DNA-directed RNA polymerase POLRMT (mitochondrial RNA polymerase) mediates the synthesis of short primers and initiates nascent L-strand mtDNA synthesis (Schwinghammer et al. 2013).

In the strand-displacement replication model, the molecular mechanisms of the mtDNA replicative process are simply divided into four steps (**Figure 7**): (1) the replisome formed by  $POL\gamma$  and TWINKLE synthesizes nascent H-strand after initiation at the  $O_H$ . The parental unwinded H-strand is stabilized by mtSSB (**Figure 7A**); (2) a stem-loop structure is formed when the H-strand replication passes through the  $O_L$  region. POLRMT binds to the stem-loop structure and synthesizes short primers that initiate the synthesis of the nascent L-strand (**Figure 7B**); (3)  $POL\gamma$  synthesizes the nascent L-strand and H-strand separately using parental H-strand and L-strand as a template (**Figure 7C**); (4) replication is terminated at either the  $O_H$  or  $O_L$  site after completion of both nascent strands (**Figure 7D**) (Bogenhagen and Clayton 2003; Bowmaker et al. 2003; Clayton 1982, 2003).



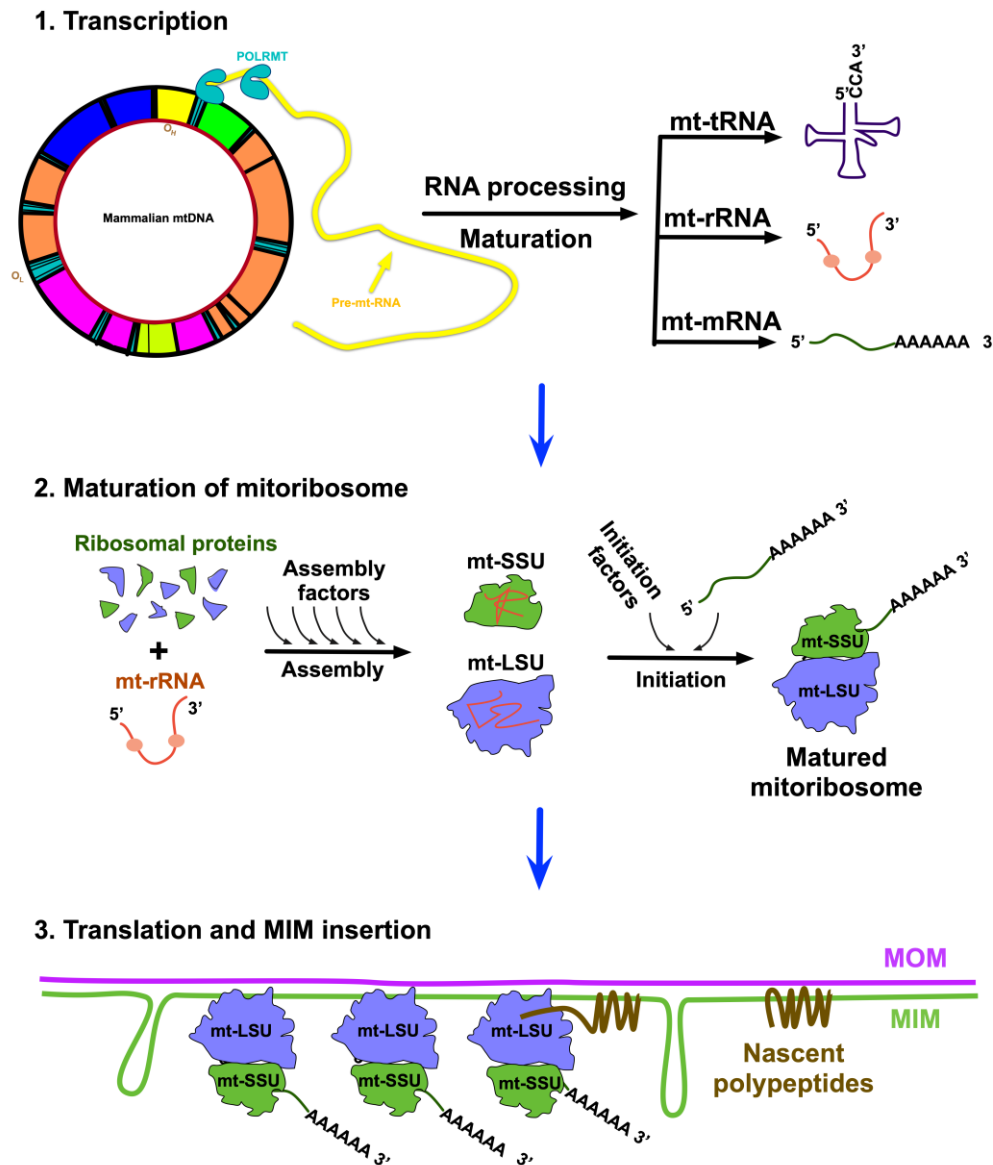


**Figure 7. Replication process of mtDNA based on the strand-displacement replication model.** (A) POL $\gamma$  (green) and TWINKLE (orange) form the replisome and unidirectionally synthesize the nascent H-strand. (B and C) The parent H-strand forms a stem-loop structure and POLRMT (light teal) synthesizes short primers (yellow) that initiates the synthesis of nascent L-strand by POL $\gamma$ . (D) The synthesis of the nascent strand (dotted line) is terminated when the initial site of replication is encountered. This picture is modified from (Shadel 2008)

## 9. mtDNA-encoded gene expression

Similar to nuclear-encoded gene expression, the process of mtDNA-encoded gene expression comprises transcription, assembly of mitoribosomes, translation, and MIM insertion of nascent polypeptides (Pearce et al., 2017; Rackham, Mercer, & Filipovska, 2012) (**Figure 8**). However, except for tRNA and rRNA, all enzymes required for mtDNA-encoded gene expression, subunits of mitoribosomes and the corresponding assembly factors are synthesized in the cytosol and imported into the matrix. Therefore, the whole process of mtDNA-encoded gene expression is controlled by dual gene expression systems. The precise regulation of gene synthesis and degradation of mtDNA-encoded RNAs determine the steady-state levels of mtDNA-encoded proteins. The fine quality control of mtDNA-encoded gene expression enables cells to adapt to various environments and

produces the necessary energy. Defects in mtDNA-encoded gene expression damage the assembly of respiratory chain complexes, which reduces ATP production and causes a multi-system disease phenotype, predominantly affecting muscular and neuronal tissues (D'Souza and Minczuk 2018).



**Figure 8. Overview of human mtDNA-encoded gene expression.** The main proteins involving in mtDNA-encoded gene expression are listed in each process. This picture is modified from Souza and Minczuk 2018; Kayal, Bentlage, et al 2016; Rackham and Mercer et al 2012.

### 9.1 Transcription of mtDNA

Transcription of the mitochondrial genome starts at the L-strand promoter (LSP) and H-strand promoter (HSP) in the D-loop region. At the initial step of transcription, the mitochondrial transcription factor A (TFAM) binds to the transcription site and introduces a 180° bend in mtDNA (Campbell, Kolesar, and Kaufman 2012; Kanki and Ohgaki 2004). Then the DNA-dependent RNA polymerase POLRMT interacts with DNA around the transcription site and with the upstream region of the TFAM-binding site. The interaction of POLRMT with mtDNA induces the conformation

change of POLRMT, which assembles the mitochondrial transcription factor B2 (TFB2M) and forms an initiation complex (McCulloch, Seidel-Rogol, and Shadel 2002; Posse and Gustafsson 2017). The initiation complex induces opening of the promotor and synthesizes RNA. The mitochondrial transcription elongation factor (TEFM) is required for the elongation of transcription via interacting with the catalytic C-terminal part of POLRMT (Schwinghammer et al. 2013). After accomplishing transcription, the mitochondrial termination of factor 1 (MTERF1) bends the mtDNA and mediates transcription termination through base flipping and DNA unwinding (Jiménez-Menéndez et al. 2010; Yakubovskaya et al. 2010). The mt-mRNA and mt-rRNA sequences are separated from the primary transcripts through excision of mt-tRNA performed by RNAase P and RNase Z at the 5'- and 3'-end, respectively (S Anderson et al. 1981; Holzmann et al. 2008; Ojala, Montoya, and Attardi 1981).

## **9.2 Translation of mtDNA-encoded proteins**

### **9.2.1 Assembly of mitochondrial ribosomes**

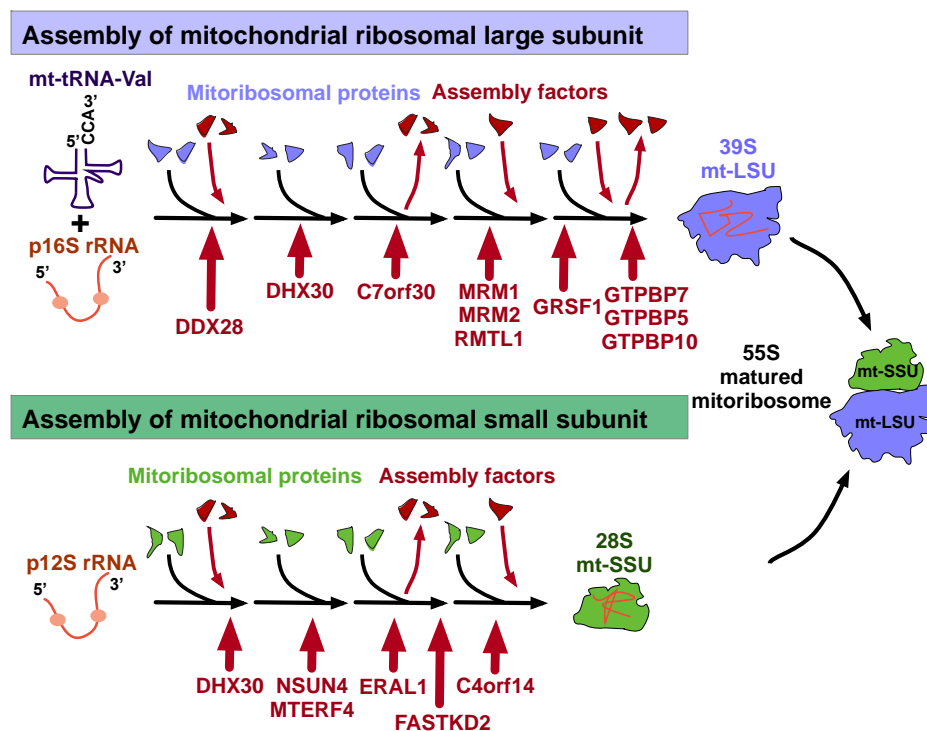
All polypeptides are translated in the ribosome using the mRNA as a template. Consistent with the cytosolic ribosome, the mature mitochondrial ribosomes comprise large ribonucleoprotein complexes composed of a large subunit (mt-LSU) and a small subunit (mt-SSU). The mt-SSU binds mRNA and translates the encoded message by selecting cognate aminoacyl-tRNA molecules (Ogle et al. 2001; Schmeing and Ramakrishnan 2009). The mt-LSU catalyzes the formation of peptide bonds through its polytidyl transferase center (PTC), thereby polymerizing the amino acids into a polypeptide chain (Ban 2000; Beringer and Rodnina 2007; Polikanov, Steitz, and Innis 2014). The mt-LSU also contains an exit tunnel that allows the newly synthesized polypeptides to leave the mitoribosomes (Jenni and Ban 2003; Kramer et al. 2009). Even though the mitochondrial ribosome is evolutionarily derived from the bacterial ribosome, mitoribosome has strongly diverged from its ancestral bacterial ribosome in terms of function, structure, rRNA and nucleoprotein composition. For example, the mitochondrial ribosome has half the length of rRNA and 36 additional nucleoproteins in comparison with the bacterial ribosome (Greber and Ban 2016; Jenni and Ban 2003; Sharma et al. 2003).

Mammalian mitoribosomes sediment as a 55S particle, which consists of a 28S mt-SSU and a 39S mt-LSU. The mt-SSU is formed by a 12S rRNA and 30 mitochondrial ribosomal proteins (MRPs) and mt-LSU is formed by a 16S rRNA and 52 MRPs (Greber and Ban 2016). All mt-rRNAs are transcript from mtDNA genes, while all 82 MRPs are nuclear gene-encoded proteins. Therefore, MRPs are synthesized in the cytosolic ribosome and imported into mitochondria during the mitoribosomal assembly process. Except for the assembly of nucleoproteins, the mt-rRNA undergoes post-transcriptional processing for the maturation of mitoribosomes (Greber and Ban 2016). The pivotal roles of mitochondrial translation for cellular energy hemostasis, biogenesis, maintenance and regulation of the complex mitochondrial translation apparatus are poorly understood. This is particularly the case regarding how the MRPs are sequentially assembled to form macromolecular structures and what proteins or cell signaling are involved in the biogenesis process. To date, only a handful of mitoribosomal biogenetic factors, rRNA modifying enzymes, RNA helicases, and GTPases, have been identified. They are transiently associated with immature mitoribosomal subunits and promote processing, modification, and stability of rRNAs and ribosomal proteins for the formation of ribonucleoprotein particles (**Figure 9**) (Barrientos, 2015; Bogenhagen, Martin, & Koller, 2014; S. S. Chen & Williamson, 2013).

In mammalian mitochondria, modification of rRNA is a necessary step for mitoribosomal biogenesis. Mitochondrial transcription factor B (TFB1M) is a methyltransferase-mediated dimethylation of two highly conserved adenosines at the 3'-end of mitochondrial 12S rRNA. This process is necessary for assembly of the mt-SSU (Metodiev et al. 2009). Another mitochondrial rRNA methyltransferase, NSUN4, methylates C911 in 12S rRNA, performing a key task in creating mature, functional mitoribosome (Metodiev et al. 2014a).

For the biogenesis of the mitoribosomal large subunit, several proteins have been identified in the assembly process. The Asp-Glu-Ala-Asp (DEAD)-box protein DDX28 promotes mt-LSU biogenesis at the early stages through interacting with 16S rRNA and the mt-LSU (Tu and Barrientos 2015a). The mitochondrial GTPase 1 and 2 (MTG1/GTPBP7) bind to the 16S rRNA to facilitate incorporation of late-assembly proteins. Through coupling of mt-LSU assembly with intersubunit bridge formation, MTG1 controls mitochondrial translation (Kim and Barrientos 2018). Furthermore, another two GTPases, MTG2/GTPBP5 and GTPBP10, are specifically associated with the mitoribosomal large subunit at a late maturation state (Hirano et al. 2006; Lavdovskaia et al. 2018; Maiti et al. 2018). In addition, the domain of unknown function 143 (DUF143) family protein C7orf30 interacts with the uL14m, promoting uL14m incorporation into mt-LSU, hence sustaining mitochondrial translation (Rorbach, Gammage, and Minczuk 2012; Wanschers et al. 2012).

For the assembly of mt-SSU, the mitochondrial GTPase C4orf14 is involved in the biogenesis of mt-SSU via an interaction with the mitochondrial nucleoid (He et al. 2012). GTP-binding protein ERAL1 is a mitochondrial RNA chaperone involved in the assembly of the 28S mt-SSU. Loss of ERAL1 leads to decreased mitochondrial translation, causing redistribution of mitoribosomal small subunits and reduced 12S rRNA (Dennerlein, Rozanska, Wydro, Zofia M. A. Chrzanowska-Lightowlers, et al. 2010; Uchiumi et al. 2010).



**Figure 9. A schematic overview of mammalian mitochondrial ribosome assembly.** Only some representative ribosomal assembly factors are shown in the pictures. The assembly stage at which they act is depicted only approximately. This picture is modified from De Silva et al. 2015.

### 9.2.1.1 rRNA-modifying enzymes

The modification of rRNA at the conserved region, usually in the catalytic domain, is a key step in ribosome assembly. These modifications can happen co-transcriptionally, immediately after transcription, or during the pre-ribosomal particle assembly process (Venema and Tollervey 1999). Compared with more than 30 rRNA modifications in the prokaryotic ribosome and 200 rRNA modifications in the eukaryotic cytoplasmic ribosome, the mitochondrial rRNA has scarcely been modified. Until now, there are three major types of rRNA modifications in mammalian mitochondria: pseudouridylation, base methylation, and 2'-O-ribose methylation (De Silva et al. 2015).

Although the enzyme responsible for pseudouridylated modification has not been identified to date, the human 16S rRNA has one pseudouridylated base at U1397 according to human 16S rRNA numbering (Motorin and Helm 2011). In mammalian mitochondria, methylation via specific methyltransferase (MTase) is the common type of rRNA modification (Motorin and Helm 2011). NSUN4 is a mitochondrial cytosine MTase that methylates cytosine 911 in 12S rRNA of the mt-SSU. In addition, NSUN4 also forms a complex with MTERF4 which is required to assemble the mt-LSU and mt-SSU (Cámara et al. 2011; Metodiev et al. 2014b). For 2'-O-ribose methylation, there are three sites in 16S rRNA of the mt-LSU that are modified by three methyltransferases, mitochondrial rRNA methyltransferase 1(MRM1), MRM2 and RMTL1 (also called MRM3). MRM1 catalyzes Gm residues at Gm1145. MRM2 and RMTL1 separately catalyze Um1369 and Gm1370, respectively, which lie in the A-loop of 16S rRNA (Lee and Bogenhagen 2014). Depletion of some of these rRNA-modifying enzymes leads to defective biogenesis of the mitoribosome, and consequently, to a deficiency in mtDNA-encoded protein level (Rorbach et al. 2014).

### 9.2.1.2 RNA helicases

RNA helicases have a structurally conserved helicase core containing characteristic sequence elements and structural motifs. These proteins bind and remodel RNA and RNA-protein complex (RNP) in an ATP- or NTP-dependent manner. The DEAD-box proteins belong to superfamily 2 RNA helicase and are commonly involved in RNA-mediated processes, RNA folding steps, RNA conformational transitions, and among others. The DEAD-box proteins were named based on the unique amino acid sequence Asp-Glu-Ala-Asp in the motif II. Unlike the conventional RNA helicases which unwind helices by translocating along one of the strands and have high processivity, the DEAD-box helicases unwind duplexes and remodel RNA-protein complexes locally within the strands and have very low processivity (Pan and Russell 2010). DDX28 is one of recently identified DEAD-box proteins that is essential for 16S rRNA stability during the early stage of mt-LSU biogenesis (Tu and Barrientos 2015b). Another RNA helicase, DHX30, is involved in all stages of mitoribosomal assembly due to extensive interaction with mt-LSU, mt-SSU, and monosomes (Antonicka and Shoubbridge 2015a).

### 9.2.1.3 GTPases

GTPases are the members of the G-protein superfamily, which is a key regulator of many cellular processes (Hall 1998; Just and Peränen 2016). Through hydrolyzing GTP, GTPases act as a major signaling mechanism involved in many critical functions, such as translation of mRNA, cell cycling, and cell division (Sprang 1997; Vetter and Wittinghofer 2001). The structure of the GTP-binding domain of all GTPases are evolutionarily conserved. The GTP-binding domain is composed of 4-5 highly conserved motifs (G1-G5), which form a characteristic fold (Pan and Russell 2010). GTPases play pivotal roles in the mitoribosomal assembly process (Nomura 1973; De Silva et al. 2015).

Two GTP-binding proteins, GTPBP5 and GTPBP7, localize to the matrix side of the mitochondrial inner membrane. GTPBP7 also known as Mtg1 specifically binds to the premature large subunit of the mitochondrial ribosome in a GTP-dependent manner and is released from the mature mt-LSU (Kotani et al. 2013a). The mt-LSU stimulates the GTPase activity of GTPBP7. The depletion of GTPBP7 reduces the overall mitochondrial translation activity and leads to the defects in the formation of respiratory complexes. GTPBP5 also known as ObgH1 belongs to the Obg family specifically associated with the mitoribosomal large subunit. The loss of GTPBP5 induces the specific activation of the translation of subunits of Complex V and disruption of its proper formation (Kotani et al. 2013b). Another protein of Obg family, GTP-binding protein 10 (GTPBP10), physically interacts with 16S rRNA and crosslinks with several proteins of mt-LSU. Due to the crucial role for mitoribosomal maturation, the deletion of GTPBP10 leads to the absence of the 55S monosome, completely inhibiting the synthesis of mtDNA-encoded proteins (Maiti et al. 2018).

Another GTPase C4orf14 is a homolog to bacterial Mtg3 which belongs to the YqeH protein family. Through transiently interacting with the mt-SSU, C4orf14 recruits specific MRPs to the 28S mt-SSU. Therefore, knockdown of C4orf14-affected components of mt-SSU leads to decreased mitochondrial protein synthesis. In addition, C4orf14 is capable of binding to DNA in vitro and loss of the C4orf14 causes a depletion of mtDNA. The association of C4orf14 with both mitochondrial translation factors and the mitochondrial nucleoid indicate that the 28S mt-SSU could be assembled at the mitochondrial nucleoid, enabling the efficiently transfer of mRNA from the nucleoid to the mitoribosome (Kotani et al. 2013b).

In mammals, another GTP-binding protein, GTPBP8, with an unknown function was found. GTPBP8 belongs to the P-loop NTPase superfamily and is a homolog to YihA in *Escherichia coli* (*E. coli*) (Leipe et al. 2002). The deletion of YihA in *E. coli* resulted in a growth rate reduction correlating to decreased levels of the cell division protein FtsZ and eventually displayed filamentation morphology, suggesting the key function in prokaryotic cells (Dassain et al. 1999). Moreover, genetic analysis also suggests the *Bacillus subtilis* (*B. subtilis*) YsxC gene, the GTPBP8 homolog in *B. subtilis*, is essential for the survival of the microorganism. Loss of YsxC protein causes the accumulation of immature ribosomal-subunit intermediates with the absence of three ribosomal proteins, uL16, bL27 and bL36 (Schaefer et al. 2006). Purified YsxC was also shown to bind preferentially to the 50S subunit ribosomes in the presence of GTP (Wicker-Planquart et al. 2008). These studies suggested that YsxC is required for the biogenesis of ribosomal large subunit.

In addition to three conserved GTPases, another conserved GTP-binding protein with RNA-binding



activity is ERAL1, which is important for the formation of 28S mt-SSU. ERAL1 localizes in the mitochondrial matrix and is involved in the biogenesis of mt-SSU through binding to the 12S rRNA. The depletion of ERAL1 leads to a rapid decay of nascent 12S rRNA (Dennerlein, Rozanska, Wydro, Zofia M. A. Chrzanowska-Lightowlers, et al. 2010; Uchiumi et al. 2010).

#### **9.2.1.4 Other mitoribosomal assembly factors**

One of the proteins in the DUF143 family, C7orf30, participates in mt-LSU assembly via interaction with uL14m, and promoting its incorporation into mt-LSU (Fung et al. 2013; Rorbach, Gammage, and Minczuk 2012; Wanschers et al. 2012). The depletion of C7orf30 diminishes the mt-LSU level and decreases the formation of monosome, eventually leading to the defective in mitochondrial protein translation (Fung et al. 2013; Rorbach et al. 2012; Wanschers et al. 2012). The m-AAA protease belongs to a conserved family of membrane-bound ATP-dependent proteases. The component of mitochondrial ribosomal large subunit, bL32m, is a proteolytic substrate of m-AAA protease. The processed product of bL32m assists in its correct folding for completely importing into pre-ribosomal particles at the late stages of mt-LSU maturation (Nolden et al. 2005). In addition, two components of mitochondrial RNA granules, G-rich sequence binding factor 1 (GRSF1) and Fas-activated serine-threonine kinase 2 (FASTKD2), are required for the assembly of mt-LSU and mt-SSU, respectively (Antonicka et al. 2013; Antonicka and Shoubridge 2015b; Jourdain et al. 2013).

#### **9.2.2 Translation**

Translation occurs by a universally conserved set of stages that can be sequentially divided into initiation, elongation, and termination in conjunction with recycling. Each step is fully mediated by various nuclear-encoded regulatory proteins. At the initiation step, mitochondrial initiation factor 3 (IF3<sub>mt</sub>) binds to ribosomal monosomes, promoting the dissociation of mitoribosome into subunits, and prevents their re-association (Koc and Spremulli 2002). Then GTP-bound IF2<sub>mt</sub> facilitates the codon-directed placement of fMet-tRNA in the P site of the ribosomal small subunits (Liao and Spremulli 1990). Following this step, IF3<sub>mt</sub> and IF2<sub>mt</sub> are sequentially released for the biogenesis of the monosome. During the elongation step three elongation factors (EFs) assist the decoding of the mRNA coding sequence. EF-Tu<sub>mt</sub> delivers the aminoacyl-tRNA to the monosome in a ternary complex with GTP. EF-Ts<sub>mt</sub> regenerates the GTP-bound form of EF-Tu<sub>mt</sub> after the guanosin diphosphate (GDP)-bound state (Schwartzbach and Spremulli 1989). EF-G1<sub>mt</sub> catalyzes the coordinated movement of mRNA and tRNA during the translocation process (Bhargava, Templeton, and Spremulli 2004). When a stop code enters the A site, the elongation is stopped by the binding of mitochondrial release factor RF1<sub>mt</sub> for release of the nascent chain. Finally, the mature monosome become a substrate for the mitochondrial ribosome recycling factor (RRF1<sub>mt</sub>), which splits mitoribosome into individual subunits so that they can reenter the translation cycle (Christian and Spremulli 2012a; Tsuboi et al. 2009).

## AIMS OF THE STUDY

Quality control of mitochondrial structure and function is paramount for critical cellular processes, from energy production to apoptosis. Notably, numerous biological processes in mitochondria, including maintenance of ultrastructure, protein expression, and regulatory functions, are almost always carried out by proteins organized further into high-ordered structures and networks. Therefore, the mitochondrial proteins with unknown functions interest us to elucidate their sub-mitochondrial localization and biological functions. Prior to the research conducted for this thesis, it was known that BAR domain proteins sculpt phosphoinositide-rich membranes to generate membrane protrusion or invaginations. Through forming dimers with a positively charged surface, BAR domain proteins bind to negatively charged lipid membranes, and thus, play crucial roles for many cellular processes involved in membrane remodeling and dynamics such as endocytosis, cell migration, and cell division (Zhao et al. 2013; Zhao, Pykäläinen, and Lappalainen 2011). However, whether some BAR domain proteins sculpt membranes in mitochondria remains obscure. Furthermore, while great strides have been made in recognizing the process of mitochondrial gene expression, the precise regulation factors involved in the formation of mitochondrial ribosomes are poorly understood. Another novel GTP-binding protein, GTPBP8, belongs to the P-loop NTPase superfamily. The deletion of bacterial homologs of GTPBP8, namely YihA in *E. coli*, has resulted in a growth rate reduction and filamentation (Dassain et al. 1999), suggesting key roles of GTPBP8 in mitochondria. To date, the detailed biological function of GTPBP8 in mitochondria remains to be clarified. Therefore, the aim of this study was to determine the biological functions of novel proteins residing in mitochondria. By elucidating functions of novel mitochondrial proteins, we can comprehend how mitochondrial activities and mitochondrial protein involved in cellular function control cell fate. This is particularly important in view of the pivotal roles of mitochondria in energy provision and cell signaling, especially in uncontrolled cancer cells. Thus, drugs can be designed to target mitochondria or some cancer-relevant mitochondrial proteins for efficient cancer therapy.

Specific aims of this project included the following:

1. Identify previously uncharacterized novel mitochondrial proteins.
2. Examine the expression profiles of novel mitochondrial proteins in cells and mouse/rat tissues. Determine the sub-mitochondrial localization of novel mitochondrial proteins by cell biology and biochemical approaches.
3. Elucidate the cellular function of novel mitochondrial proteins and the underlying molecular mechanisms.
4. Explore whether mitochondria is a useful target for efficient cancer therapy, providing some experiment hints for solving current problems of cancer therapy.



## MATERIALS AND METHODS

Table 1. The main experimental methods applied in this study. The details of the materials, including antibodies, reagents and kits, and methods used in the study can be found in the corresponding articles and the affiliated supplementary information.

Methods	Publications
Mammalian cell culture and transfection	I, II, III
Transfection for protein knockdown and expression	I, III
Rescue experiments	I, III
CRISPR/Cas9-mediated gene knockout	I
Immunofluorescence	I, III
APEX2 staining	I
ImmunoEM	I, III
Transmission electron microscopy	I, II, III
Electron cryotomography	I
Time-lapse image of cells	I
Fluorescence light microscopy	I, II, III
SDS-polyacrylamide gel electrophoresis	I, II, III
Blue-native polyacrylamide gel electrophoresis (BN-PAGE)	I, III
Western blotting	I, II, III
Molecular cloning and site-directed mutagenesis	I, III
DNA and RNA isolation	III
Real-time quantitative PCR	III
Production and purification of recombinant Proteins	I, III
Mitochondrial complex I and IV activity assay	I
GTPase activity assay <i>in vitro</i>	III
Vesical preparation	I
Membrane binding assay	I
Cell fractionation assay	I, III
Mitochondrial isolation	I, III
Proteolysis of mitochondria and alkaline extraction	I, III
Cell viability assay	I, III
Detection of mitochondrial membrane potential, ROS and ATP	I, III
Mass spectrometry	III
Measurement of cellular metabolism	I, III
Radioisotope labeling	III
Isokinetic sucrose gradient assay	III
Quantitative western blotting or coomassie stained SDS PAGE analysis	I, III
Quantitative image analysis (EM data)	I

### **Immunofluorescence, live-cell imaging and ImmunoEM**

For immunofluorescence, U2OS cells were grown on coverslips and fixed with 4% paraformaldehyde for 20 min, washed three times with PBS and permeabilized for 5 min with 0.1% Triton X-100 in PBS. For antibody staining, permeabilized cells were blocked with Dulbecco plus 0.2% BSA for 30 min and were sequentially incubated with primary antibody and fluorescence-conjugated secondary antibody. Cells were mounted in Mowiol containing 2.5% wt/vol 1,4-diazabicyclo[2.2.2]octane (DABCO) and imaged with DM6000B microscope using Application of Suite X software using 63x/1.4-0.60 HCX PL Apochromat objective, and brightline filters (Semrock): GFP-4050B (excitation, 466/40; emission, 525/50), TRITC-B (excitation, 543/22; emission, 593/40), with an Orca-Flash4.0 V2 scientific complementary metal-oxide semiconductor (sCMOS) camera (Hamamatsu Photonics).

For live-cell imaging, U2OS cells were transfected mitochondrial matrix targeted YFP (mito-YFP) for 48h. Cells were re-plated on 10 µg/ml fibronectin-coated glass-bottomed dishes in complete DMEM medium. Put the dishes in a 37°C sample chamber with controlled 5% CO<sub>2</sub> flow and the time-lapse images were acquired with 3I Marianas imaging system, equipped with an inverted spinning disk confocal microscope Axio Observer Z1 (Zeiss) and a Yokogawa CSU-X1 M1 confocal scanner (Yokogawa) and brightline filter (Semrock). A 63x/1.2 W C-Apochomat Corr WD =0.28 M27 objective was used and all the images were acquired by a sCMOS 34 Neo camera (Andor) and slideBook 5.0 software. Analyses of the video frames were performed with Image Pro Plus 6.0 (Media Cybernetics).

For pre-embedding immunoEM, U2OS cells were grown on coverslips and fixed with PLP fixative (2% formaldehyde, 0.01 M periodate, and 0.075 M lysine-HCL in 0.075 M phosphate buffer, pH 7.4) for 2 h (Uchiyama et al. 2002). Cells were permeabilized with 0.05% saponin and immunolabeled using primary antibody followed by immunostaining with Nanogold secondary antibody. Nanogold was silver enhanced using the HQ Silver kit for 5 min and gold toned with 0.05% gold chloride. After washing, the cells were further processed for embedding.

### **Isolation and purification of mitochondria**

Mitochondria were isolated and purified as described previously (Frezza, Cipolat, and Scorrano 2007). Briefly, cells were washed in precold PBS and resuspended in homogenization buffer (10 mM Tris-MOPS, 1 mM EGTA, and 200 mM sucrose, pH 7.4). Cell suspension was homogenized with a rotating Teflon potter (Potter S; Braun). Nonlysed cells were sedimented at 600 g and discarded. The supernatant was subsequently centrifuged at 7,000 g, and the resulting pellet was the mitochondrial fraction.

### **Proteolysis of mitochondria and Alkaline extraction of mitochondrial proteins**

For proteolysis of mitochondria, mitochondria were subfractionated to obtain mitoplasts by using a phosphate swelling-shrinking method (Kang et al. 2007). Briefly, for breaking the MOM, purified mitochondrial pellets were suspended in swelling buffer (10 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4) and incubated for 20 min with gentle mixing. To keep the mitoplasts intact, mitochondria were mixed with equal volume of shrinking buffer (10 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4, 32% sucrose, 30% glycerol, and 10 mM MgCl<sub>2</sub>) for another 20 min. The purified mitochondria and mitoplasts were suspended in homogenization

buffer (10 mM Tris-MOPS, 1 mM EGTA, and 200 mM sucrose, pH 7.4) and treated with 0.2 mg/ml proteinase K with or without the presence of 1% NP-40 for 30 min. NP-40 was used to gently permeabilize mitochondrial membranes to allow proteinase K to enter mitochondria. Proteinase K activity was quenched with 2 mM PMSF for 10 min. All steps were performed on ice. 1% SDS was added to solubilize mitochondrial proteins, and the samples were blotted as indicated.

For alkaline extraction of mitochondrial proteins, the purified mitochondria were resuspended in 0.1 M Na<sub>2</sub>CO<sub>3</sub>, pH 11, 11.5, or 12, or PBS, pH 7.4, on ice for 30 min with occasional vortex mixing and then centrifuged at 51,000 rpm for 30 min at 4°C using an Optima ultracentrifuge with a TLA 120 rotor (Beckman Coulter). The membrane pellets were dissolved in Laemmli loading buffer (2% SDS, 10% glycerol, 60 mM Tris-HCL, and 0.005% bromophenol blue). Supernatants were precipitated using TCA with a final concentration of 13% on ice for 30 min. After centrifugation at 20,000 g for 30 min, pellets were washed two times with ice-cold acetone and dissolved in Laemmli loading buffer. The initial total mitochondria used for alkaline extraction, pellets, and supernatant were subjected to SDS-PAGE and Western blotting analysis.

### **Blue-native PAGE**

The blue native gel electrophoresis was performed using the NativePAGETM Novex® Bis-Tris Gel System according to manufacturer's protocol. Briefly, mitochondria were suspended in lysis buffer (50 mM Bis-Tris, 50 mM NaCl, 10% glycerol, 0.001% Ponceau S, 1.2% digitonin, 16 mM HCL, pH 7.2) on ice for 30 min and centrifuged at 22,000g for 30 min. The supernatant was supplemented with 0.2% Coomassie G250 dye and loaded on a 3-12% gradient NativePAGE Gel. Proteins were transferred to the polyvinylidene fluoride (PVDF) membrane which was subsequently incubated in 8% acetic acid and activated with methanol.

### **Measurement of cellular metabolism**

The cellular oxygen consumption rate (OCR) was measured using a Seahorse XF96e analyzer (Seahorse Bioscience; Agilent Technologies). The XF96 sensor cartridge was activated with 200 µl XF96 calibrant solution per well for 12 h at 37°C. U2OS cells were seeded onto XF96 cell culture microplates and change the culture medium with serum-free and bicarbonate-free DMEM supplemented with 10 mM glucose, 5 mM pyruvate, and 5 mM glutamine one hour before measurement. After incubation for 1 h at 37°C in a non-CO<sub>2</sub> incubator, steady-state and postintervention analyses were performed. Respiration was assessed by injection of oligomycin (1 µM) to inhibit the mitochondrial ATP synthase, carbonyl cyanide-p-trifluoromethoxy-phenylhydrazone (FCCP; 1 µM) to collapse the mitochondrial membrane potential, and rotenone (1 µM) and antimycin A (1 µM) to inhibit the respiratory chain. The OCR was measured before and after the addition of inhibitors at indicated times. The OCR was normalized to total protein amount (micrograms).

### **Measurement of mitochondrial membrane potential, ROS and ATP production**

Mitochondrial membrane potential was measured using the fluorescent dye tetramethylrhodamine methyl ester (TMRM; T668; Thermo Fisher Scientific). U2OS cells were stained with 50 nM TMRM for 15 min at 37°C. Cellular superoxide anions and mitochondria superoxide were detected using 10 µM dihydroethidium (DHE, D1168; Thermo Fisher Scientific) and 1 µM MitoSOX (M36008;

Molecular Probes), respectively. Cellular ATP levels were detected by an ATP determination kit (A22066; Thermo Fisher Scientific) according to the manufacturer's instructions. The fluorescence intensity was recorded using a Varioskan LUX multimode microplate reader with SkanIt Software 5.0 (Thermo Fisher Scientific). The excitation (ex) and emission (em) wavelength were set basing on instruction of each fluorescent dye with a slightly modify. TMRM: ex549nm/em580nm; DHE: ex510nm/em600nm; MitoSOX: ex510nm/em580nm;

### **Radioisotope labeling of mitochondrial translation**

Mitochondrial protein synthesis in cultured cells was detected through metabolic labeling with [<sup>35</sup>S] methionine/cysteine in the presence of anisomycin to inhibit cytoplasmic ribosomes (Richter et al. 2015). Simply, U2OS cells were washed three times with PBS and pretreated with 100 µg/ml anisomycin for 5 min to inhibit cytoplasmic translation. Subsequently, [<sup>35</sup>S] methionine/cysteine (EasyTag; PerkinElmer) was added with a final concentration of 400 µCi for pulse labeling. After pulse labeling for indicated time, cells were washed two times with PBS for detection or medium without [<sup>35</sup>S] methionine/cysteine for chase labeling. After 6 h chasing, cells were scraped and treated with benzonase<sup>®</sup> Nuclease (E1014, Sigma) according to the manufacturer's instructions. The samples were supplemented with gel loading buffer (186-mM Tris-HCL, pH 6.7, 15% glycerol, 2% SDS, 0.5 mg/ml bromophenol blue, and 6% β-mercaptoethanol) and loaded on a 12-20% gradient SDS-PAGE gel to separate samples. The gel was then dried for exposure with a phosphor screen and scanned with a FUJIFILM FLA-5100. Gels were rehydrated in water and Coomassie stained to confirm loading.

### **Isokinetic sucrose gradient assay**

The sedimentation properties of GTPBP8 and the ribosomal proteins in sucrose gradients were analyzed essentially as described (Richter et al. 2015). Cells or mitochondria were lysed with 1% DDM lysis buffer (50 mM Tris, pH 7.2, 10 mM Mg(Ac)<sub>2</sub>, 40 mM NH<sub>4</sub>Cl, 100 mM KCl, 1% DDM, and 1 mM PMSF) for 20 min on ice followed by centrifugation for 20 min at 20,000 g and 4°C. The supernatant containing 900 µg total protein was loaded on top of a 16 ml linear 10-30% sucrose gradient (50 mM Tris, pH 7.2, 10 mM Mg(Ac)<sub>2</sub>, 40 mM NH<sub>4</sub>Cl, 100 mM KCl, and 1 mM PMSF) and centrifuged for 15 h at 4°C and 74,400 g (SW 32.1 Ti; Beckman Coulter). 24 equal volume fractions were collected from the top and subjected to TCA precipitation. Samples were separated by SDS-PAGE for immunoblotting.

## RESULTS AND DISCUSSION

### 10. Cellular localization of FAM92A1 and GTPBP8

#### 10.1 Identification of novel proteins FAM92A1 and GTPBP8

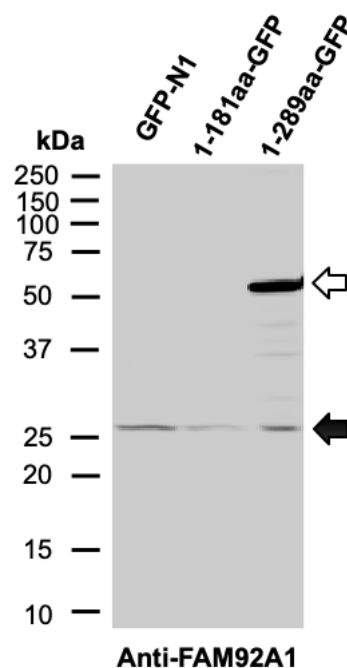
The BAR domain proteins play key roles in membrane remodeling (Zhao et al. 2013, 2011), but none are involved in the remodeling of mitochondrial membrane structure, especially the high curved crista structure. First of all, we searched for novel BAR domain protein in the conserved BAR domain database by NCBI combined with BLAST searches using the classical BAR domain of endophilin. We found an uncharacterized BAR domain protein FAM92A1, which is highly conserved among mammals and contains a classical BAR domain at the N-terminus (Carman and Dominguez 2018). We then aimed to investigate the cellular localization and function of FAM92A1, to shed light on whether FAM92A1 is involved in sculpting the membranes where it resides and how FAM92A1 remodels membrane structures. In addition, because GTPases play important roles in membrane fission and fusion we searched for novel GTP-binding proteins by conserved GTPase domains. A novel GTP-binding protein 8, GTPBP8, was identified. We aimed to elucidate the subcellular localization and cellular function of GTPBP8.

#### 10.2 FAM92A1 and GTPBP8 are mitochondrial proteins

##### 10.2.1 Validation of commercial antibodies

The cellular localization of FAM92A1 and GTPBP8 was examined in U2OS cells. To address the above questions, commercial antibodies were used to test the cellular localization of the endogenous FAM92A1 and GTPBP8 proteins. First, the commercial anti-FAM92A1 and anti-GTPBP8 antibodies were validated using Western blotting. The antigen used for generating the anti-FAM92A1 antibody (Cat. #HPA034760, Sigma-Aldrich) is KTIFSEFITIEMLFHGKALEVYTAAYQNIQNIDEDEDLEVFRNSLYAPDYSSRLDIVRANSKSPLQRSLSAKCVSGTGQEAGWAATCQTLI (FAM92A1-Human, 181-289aa). The anti-FAM92A1 antibody displays only one band at approximately 29 kDa in the Western blotting of the U2OS cell lysates. To validate the antibody of FAM92A1, we overexpressed the recombinant green fluorescent protein (GFP)-tagged full-length human FAM92A1 (1-289aa-GFP) and the mutant 1-181aa (1-181aa-GFP). The anti-FAM92A1 antibody can recognize the full-length recombinant 1-289aa-GFP but not the 1-181aa-GFP (**Figure 10**). In addition, in the gene knockdown experiments, the specific band recognized by anti-FAM92A1 antibody was gradually depleted with extended time of FAM92A1 siRNA treatment (**I, Figure 2A**). Taken together, these data suggest that the commercial anti-FAM92A1 antibody specific recognized to the endogenous FAM92A1 protein.

**Figure 10. The endogenous FAM92A1 protein can be specific recognized by an anti-FAM92A1 antibody.** Western blotting separately detected the overexpressed GFP-N1, 1-181aa-GFP and 1-289aa-GFP. Only the full-length



FAM92A1 (1-289aa-GFP) can be visualized. The mutant lacking of antigen (1-181aa-GFP) cannot be assessed. The black and white arrow showed endogenous FAM92A1 and the expressed GFP-tagged FAM92A1 protein, respectively.

The anti-GTPBP8 antibody (Cat. #HPA03481, Sigma-Aldrich) was validated using GTPBP8-GFP and GTPBP8-myc expressed U2OS cells. The antigen used for generating the anti-GTPBP8 antibody is AGRLFEMPAVLERLSRYNSTSQAFAEVLRLPKQQLRKLLYPLQEVERFLAPYGRQDLHL RIFDPSPEDIARADNIFTATERNRIDYVSS (GTPBP8-Human 10-98aa). The anti-GTPBP8 antibody recognized several bands in the Western blotting assay. The size of the endogenous GTPBP8 is around 28 kDa (1-284aa). The overexpressed GTPBP8-myc in U2OS cells can also be detected by the anti-GTPBP8 antibody with the position a little bit higher than the endogenous GTPBP8 at around 31 kDa (**III, Figure S2**). In addition, mitochondria isolated from U2OS cells were loaded on the gel with different mitochondrial quantities. Consistent with the results of endogenous and overexpressed GTPBP8-Myc, the intensity of detected band around 28 kDa was gradually enhanced with an increased quantity of mitochondria (**III, Figure S2C**). Furthermore, the band intensity around 28 kDa was decreased after treatment with GTPBP8 siRNA (**III, Figure 2B**), suggesting that the band around 28 kDa recognized by the commercial anti-GTPBP8 antibody is the endogenous GTPBP8 protein. Taken together, these data suggested that both anti-FAM92A1 and anti-GTPBP8 antibodies can recognize the corresponding endogenous proteins.

### 10.2.2 Cellular localization of FAM92A1 and GTPBP8

The anti-FAM92A1 antibody recognized only one band in the Western blotting and our results showed that this antibody is very specific. Thus, we used the anti-FAM92A1 antibody to determine the cellular localization of FAM92A1. Due to multiple bands can be detected by anti-GTPBP8 antibody in western blotting, except anti-GTPBP8 antibody applied for endogenous GTPBP8 antibody, we further used the expressed GTPBP8-GFP and GTPBP8-myc to verify the cellular localization of GTPBP8 in U2OS cells. Fluorescence microscopy showed that both endogenous FAM92A1, GTPBP8 and expressed GTPBP8 (GTPBP8-GFP and GTPBP8-Myc) co-localized with the mitochondrial marker, MitoTracker red (**I, Figure 1B**) and mitochondrial outer membrane protein TOM20 and mitochondrial inner membrane protein COXIV (**III, Figure 1A**), respectively. The cell fractionation confirmed that the majority of the endogenous FAM92A1 (**I, Figure 1C**) and all of the endogenous GTPBP8 (**III, Figure 1C and S2D**) resided in the mitochondrial fraction. These data indicated that FAM92A1 and GTPBP8 are mitochondrial proteins.

### 10.3 Sub-mitochondrial localization of FAM92A1 and GTPBP8

Mitochondria possess two enclosed membranes with several sub-mitochondrial functional domains. Thus, to determine the sub-mitochondrial localization of GTPBP8 and FAM92A1, isolated mitochondria were treated with proteinase K in the absence or presence of detergent NP-40. Compared with outer membrane proteins TOM20 and TOM40, which were sensitive to proteinase K and degraded when treated with proteinase K, FAM92A1 and GTPBP8 were resistant to proteinase K digestion. After the outer membrane was disrupted by hypo-osmolar treatment, the mitoplasts were exposed with proteinase K. The mitochondrial intracristal space protein cytochrome C was degraded when mitoplasts were treated with proteinase K. In contrast to cytochrome C, GTPBP8 and FAM92A1 were not accessible to proteinase K, similarly to the mitochondrial inner membrane protein COXIV and the matrix protein HSP60. After complete solubilization of mitochondrial

membranes, all proteins were degraded by proteinase K. Together, these data indicated that FAM92A1 (**I**, **Figure 1D**) and GTPBP8 (**III**, **Figure 1D**) resided either in the inner leaflet of the MIM or in the matrix. To corroborate these findings, we performed immunoelectron microscopy using anti-FAM92A1 and anti-myc antibodies to determine the sub-mitochondrial localization of the endogenous FAM92A1 and expressed GTPBP8-myc, respectively. The results confirmed that both FAM92A1 and GTPBP8-myc localized in the matrix in close proximity to the inner membrane (**I**, **Figure 1E** for FAM92A1 and **III**, **Figure 1F** for GTPBP8).

Subsequently, to examine whether FAM92A1 and GTPBP8 are bound to the MIM or soluble in the matrix, the isolated mitochondria were subjected to alkaline carbonate extraction with increasing pH. The integral MIM protein COXIV, the peripheral MIM protein uL16m and the soluble matrix protein HSP60 served as controls. Interestingly, FAM92A1 was found predominately in the pellet/membrane fraction but was released to the supernatant with increasing pH, indicating that FAM92A1 is a membrane-bound but not an integral membrane protein (**I**, **Figure 1G**). In contrast to FAM92A1, the membrane binding ability of GTPBP8 was relatively weaker, and the majority of GTPBP8 protein was recovered in the supernatant fraction at pH 11, displaying a similar pattern with the mitoribosomal large subunit protein uL16m (**III**, **Figure 1E**). Taken together, our data revealed that FAM92A1 and GTPBP8 are mitochondrial proteins peripherally associated with the MIM.

#### 10.4 Mitochondrial import of FAM92A1 and GTPBP8

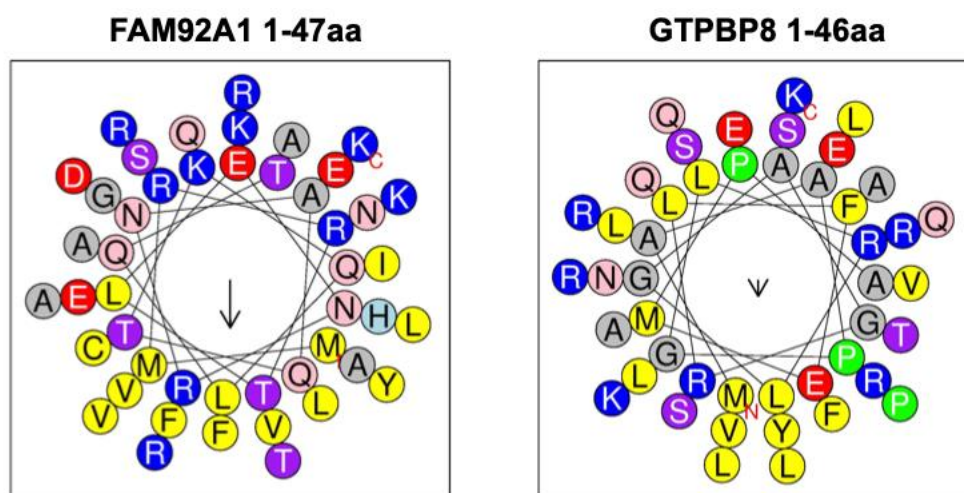
Most nuclear-encoded mitochondrial proteins are synthesized in the cytosol and imported into mitochondria by a cleavable mitochondrial targeting presequence (Bolender et al. 2008; Neupert and Herrmann 2007). The mitochondrial import presequence is classically an amphipathic helix that is 15-70 residues in length and enriched in the positively charged cluster on one side of the helix (Alberts et al. 2002; Bolender et al. 2008). To address whether FAM92A1 and GTPBP8 possess such mitochondrial targeting sequence, we analyzed their amino acid sequences and performed the online mitochondrial import prediction algorithms. The online prediction results indicate a clear mitochondrial import sequence in GTPBP8 (IMPI and TargetP score is 1 and 0.815, respectively. <http://mitominer.mrc-mbu.cam.ac.uk/release4.0/report.do?id=1021783&trail=%7c1021783>). Despite a lower possibility of mitochondrial import sequence for FAM92A1 predicted by the online program, FAM92A1 harbors a characteristic mitochondrial import presequence at the N-terminus, consisting of an alternating pattern of hydrophobic and positively charged amino acids, exhibiting a high number of arginine and lysine, with only a few negatively charged residues (**Figure 11**).

FAM92A1_Human	MMRTLENSNAQTQLQTANSNEKHGELQLEAAYVETARLEDDADLLVNEINAYAA	60
FAM92A1_Human	TETPHLKLGLMNFADFAKLQDYRQAEVERLEAKVVEPLKTYGTIVKMKRDDLKATLTAR	120
FAM92A1_Human	NREAKQLTQLERTRQRNPSDRHVISQAETELQRAAMDASRTSRHLEETINNFERQKMKDI	180
FAM92A1_Human	KTIFSEFITIEMLFHGKALEVYTAAYQNIQNIDEDLEVFNRNSLYAPDYSSRLDIVRAN	240
FAM92A1_Human	SKSPLQRSLSAKCVSGTGQVSTCRLRKDQQAEDDEDLDVTEENFLK	289
GTPBP8_Human	MAAPGRGAGRTEMPAVLERLSRYNSTSQAFAEVRLRPQQQLRLLYPLQEVERFLAP	60
GTPBP8_Human	YGRQDLHLRIFDPSPEIDIARADNIFTATERNRIDYVSSAVRIDHAPDLRPEVCFIGRSN	120
GTPBP8_Human	VGKSSLIKALFSLAPEVEVRVSKKPGHTKKMNFVKVGHFTTVVDMPGYGFRAPEDFVDMV	180
GTPBP8_Human	ETYLKERRNLKRTFLLVDSVVGIQKTDNIAIEMCEEALPYVIVLTKIDKSSKGHLLKQV	240



**Figure 11. Sequence information of human FAM92A1 and GTPBP8.** The N-terminal putative mitochondrial targeting sequences were shown in blue. The positively charged amino acids and hydrophobic amino acids were indicated with red and green, respectively.

To determine the sequence elements required for mitochondrial targeting, the N-terminal 47 amino acids (47aa) of FAM92A1 and 46 amino acids (46aa) of GTPBP8 were linked to the GFP (**I, Figure 1H** for FAM92A1 and **III, Figure 1B** for GTPBP8). Transient expression of these constructs in U2OS cells showed an unambiguous mitochondrial localization. In contrast, FAM92A1 and GTPBP8 lacking the N-terminal presequence displayed a cytosolic localization (**I, Figure 1I** for FAM92A1 and **III, Figure 1G** for GTPBP8), demonstrating that the N-terminal peptides are essential for the import of FAM92A1 and GTPBP8 into mitochondria. The mitochondrial targeting peptides usually form amphipathic  $\alpha$  helices, allowing the proteins to interact with membranes. To identify whether the putative presequence of FAM92A1 1-47aa and GTPBP8 1-46aa generates a helical structure, the helical wheel projection diagrams for both peptides were executed using Heliquest software (Gautier et al. 2008). The wheel projections strongly suggested the formation of an  $\alpha$  helix structure as shown in **Figure 12**. Taken together, the N-terminus of both proteins are essential for mitochondrial import.



**Figure 12. Helical wheel projections of putative mitochondrial targeting sequence.** Helical projections of  $\alpha$ -helices were obtained using the HeliQuest web server (<http://heliquest.ipmc.cnrs.fr/>). Residues are color-coded in blue (K, R) for positively and red (D, E) for negatively charged residues. Non-polar residues are shown in yellow or gray and others in purple, light blue, or light pink. The arrow shows the hydrophobic moment.

In addition, the putative molecular weight of FAM92A1 and GTPBP8 is around 32 kDa and 31 kDa, respectively. However, the actual detected molecular weight of FAM92A1 and GTPBP8 in U2OS cells were at 29 kDa and 28 kDa, respectively, suggesting that the N-terminal presequence of both proteins are cleaved off after mitochondrial import.



## **11. Function characterization of FAM92A1 and GTPBP8**

### **11.1 Depletion of FAM92A1 and GTPBP8 results in reduced cell proliferation**

To gain insight into the function of FAM92A1, the CRISPR/Cas9-based method was applied to knockout FAM92A1 protein. However, knockout of FAM92A1 was lethal and no stable clone was acquired, suggesting the essential role of FAM92A1 for cell survival. Therefore, small interfering RNA (siRNA) was applied to knockdown the endogenous FAM92A1 protein in U2OS cells. The effectiveness of protein silencing was assessed after treatment with siRNA at indicated times (**I, Figure 2A**). The FAM92A1 level was gradually decreased with extended time of siRNA treatment, suggesting that FAM92A1 siRNA was specifically targeted to FAM92A1. To investigate the function of GTPBP8, the GTPBP8 siRNA pool was applied to knockdown endogenous GTPBP8 protein. The result showed two siRNA strands (2# and 4#) induced at least 90% reduction of GTPBP8 at transcription level (**III, Figure 2A**). Therefore, the siRNA 4# was applied in further studies. After treatment with siRNA 4# for 5 days, the endogenous GTPBP8 was efficiently depleted in U2OS cells (**III, Figure 2B**). Cell proliferation after the siRNA treatment was evaluated using Thiazolyl Blue Tetrazolium Bromide (MTT) assay, showing that loss of FAM92A1 and GTPBP8 resulted in low cell proliferation rate compared with control cells (**I, Figure 2B** for FAM92A1 and **III, Figure 2C** for GTPBP8). The inhibited cell growth after depletion of FAM92A1 and GTPBP8 indicates that FAM92A1 and GTPBP8 are required for cell survival.

### **11.2 Depletion of FAM92A1 and GTPBP8 increases oxidative stress in cells**

The maintenance of oxygen homeostasis in mammalian cells is fundamental for the survival of the organism. Mitochondria have been validated as the main generator of reactive oxygen species (ROS). The excessive oxidant stress burden triggers the opening of mitochondrial channels, leading to the simultaneous collapse of the mitochondrial membrane potential, which finally causes cell death through apoptosis (Zorov et al. 2000; Zorov, Juhaszova, and Sollott 2006). Therefore, to determine the molecular mechanisms involved in the reduced cell growth ability, the mitochondria generated ROS was assessed using a mitochondrial targeted fluorescent superoxide sensor (MitoSox). Compared with control cells, FAM92A1 and GTPBP8 depletion caused a significant increase in oxidative stress in mitochondria (**I, Figure 2D** for FAM92A1 and **III, Figure 2D** for GTPBP8). Furthermore, the mitochondrial membrane potential was simultaneously diminished in FAM92A1 defective cells (**I, Figure 2C**). These results suggest that the cell growth arrest caused by depletion of FAM92A1 and GTPBP8 is attributed to different mechanisms.

### **11.3 Loss of FAM92A1 and GTPBP8 impairs mitochondrial respiration**

Through protonic circuits, mitochondria have the capability to couple electron transportation and oxidative phosphorylation, generating maximal ATP for normal mammalian cell activity (Steinberg-Yfrach et al. 1998). Electron transfer from respiratory chain substrates to molecular oxygen results in the vectorial translocation of protons, and this protonic energy is utilized for ATP synthesis, ion translocation, and mitochondrial protein import (Hatefi 1985). Thus, OCR and ATP level are widely applied to evaluate mitochondrial respiration ability. In our studies, OCR was measured to examine the effects of FAM92A1 and GTPBP8 depletion on mitochondrial respiration. Strikingly, silencing of FAM92A1 and GTPBP8 induced a drastic drop in rates of oxygen consumption, indicating

significantly diminished mitochondrial respiratory capacity (**I, Figure 2F** for FAM92A1 and **III, Figure 2F** for GTPBP8). Consistent with this, the total cellular ATP levels were decreased after depletion of FAM92A1 and GTPBP8 in U2OS cells (**I, Figure 2H** for FAM92A1 and **III, Figure 2H** for GTPBP8).

#### **11.4 Depletion of FAM92A1 and GTPBP8 leads to aberrant assembly of respiratory chain complexes**

The respiratory chain complexes consist of five heteromeric complexes, complex I, II, III, IV, and V. Except for complex II, several core subunits of the respiratory chain complexes are encoded by mtDNA. Our data revealed that mitochondrial respiration and cellular ATP level were decreased after depletion of FAM92A1 or GTPBP8. We wondered whether depletion of FAM92A1 or GTPBP8 would affect the assembly of respiratory complexes. To address this question, BN-PAGE followed by immunoblotting analysis was performed to examine the assembly of respiratory chain complexes in isolated mitochondria after knockdown FAM92A1 and GTPBP8. The results showed that depletion of FAM92A1 predominantly reduced the formation of complexes I and IV (**I, Figure 2I**) as well as the corresponding enzyme activity (**I, Figure 2J**). In contrast, the assembly of respiratory chain complexes I, III, IV, and V were moderately decreased after depletion of GTPBP8 (**III, Figure 2I**). However, the assembly of nuclear-encoded complex II was not significantly affected by depletion of GTPBP8, suggesting that the aberrant assembly of the respiratory chain complexes containing mtDNA-encoded subunits may be related to the steady-state level of mtDNA-encoded subunits.

#### **11.5 Aberrant level of respiratory complexes in FAM92A1 and GTPBP8 depletion cells was caused by different mechanisms**

To determine the factors causing the abnormal respiratory complexes, the mtDNA copy number and steady-state protein level of subunits from both nuclear-encoded and mtDNA-encoded subunits were measured. The results revealed that the protein levels tested from both genomes were unaffected by FAM92A1 knockdown (**I, Figure 2K**), indicating that defects in the assembly of complex I and IV may be attributed to abnormal assembly processes or poor stability of complexes, but not to decreased expression level of protein subunits. In GTPBP8-depleted cells, the steady-state level of mtDNA-encoded proteins were significantly decreased, while the nuclear-encoded protein levels were unaffected (**III, Figure 3A**). These data imply that the defects of respiratory complexes assembly in GTPBP8-depleted cells were caused by the diminished expression levels of mtDNA-encoded subunits.

## **12. FAM92A1 regulates mitochondrial morphology and inner membrane architecture**

### **12.1 FAM92A1 depletion induces abnormal mitochondrial morphology and impairs mitochondrial dynamics**

Mitochondrial function is closely linked to organelle morphology and structure. Therefore, mitochondrial morphology and dynamics were examined after downregulation of FAM92A1 using fluorescence microscopy and living cell imaging, respectively. The results revealed that depletion of FAM92A1 induced dramatic changes in mitochondrial morphology with the appearance of abnormal spherical and fragmented mitochondria, in contrast to the tubular mitochondrial network in control

cells (**I, Figure 3A**). Furthermore, loss of FAM92A1 obviously impaired mitochondrial dynamics. The bleb-shaped mitochondria displayed clear defects in mitochondrial fusion and fission (**I, Videos 1 and 2**).

## **12.2 Loss of FAM92A1 causes aberrant mitochondrial inner membrane architecture**

To further assess the changes in mitochondrial morphology, the mitochondrial ultrastructure was examined using transmission electron microscopy. Interestingly, the results showed that FAM92A1 depletion drastically changed the mitochondrial inner membrane architecture, without apparent effects on the mitochondrial outer membrane. The majority of mitochondria in FAM92A1-knockdown cells had less membrane invagination and membrane remodeling than the elongated invaginations presenting in control cells. Some mitochondria in FAM92A1-knockdown cells were completely devoid of membrane invaginations (**I, Figure 3C and 3D**). These data indicate that FAM92A1 is involved in the maintenance of mitochondrial inner membrane architecture.

The mitochondrial inner membrane consists of two domains, the inner boundary membrane and the crista membrane, which are connected by crista junctions (Frank et al. 2001; von der Malsburg et al. 2011). To further determine which ultrastructure was directly linked to FAM92A1, the width of the crista junction opening and the length of crista were quantified based on images obtained in electron microscopy. Interestingly, depletion of FAM92A1 profoundly reduced the length of crista without significant effects on the morphology of crista junction (**I, Figure 3E and 3H**).

Despite the majority of crista junctions being unaffected, some were widened in FAM92A1 depleted cells compared with control cells (**I, Figures S2K**). Crista junctions are highly curved tubular openings that separate crista membrane invaginations from the surrounding boundary membranes. Extensive studies showed that the MICOS complex is required for the biogenesis and maintenance of mitochondrial crista junction as well as proper tethering of the two mitochondrial membranes (Barbot et al. 2015; Rabl et al. 2009). In human mitochondria, the MICOS complex is composed of six subunits forming a large complex around the crista junction. Depletion of some subunits reduces the formation of the MICOS complex and eventually leads to aberrant crista morphology (Olichon et al. 2003). To examine whether depletion of FAM92A1 affects the MICOS complex, the level of MICOS complex was determined using BN-PAGE. The results showed that the abundance of MICOS complex was decreased in FAM92A1 depleted cells relative to control cells (**I, Figure S2L**). Thus, we speculate that in FAM92A1 depleted cells the loss of membrane invagination caused by FAM92A1 depletion affects the stability of MICOS complex, causing degradation of the MICOS complex. However, in already formed membrane invaginations, the MICOS complex retained its ability to maintain normal crista junction opening.

Taken together, our data revealed that FAM92A1 is essential for maintaining mitochondrial morphology and inner membrane ultrastructure. Loss of FAM92A1 impaired mitochondrial inner membrane architecture, which led to defects in the assembly or stability of the respiratory and MICOS complexes.

### **13. FAM92A1 binds to negatively charged phospholipids generating positive membrane curvature**

#### **13.1 FAM92A1 forms dimers and binds mitochondrial membranes**

The BAR domain superfamily proteins are widely known for their involvement in membrane remodeling in various cellular pathways ranging from endocytic vesicle and tubule formation to cell migration and neuron morphogenesis (Dawson et al. 2001; Dharmalingam et al. 2009; Ho et al. 2004; Kim et al. 2008; Krugmann, Jordens, and Gevaert 2001). Depletion of FAM92A1 caused profound changes in mitochondrial inner membrane ultrastructure. Thus, we aim to examine whether FAM92A1 is capable of binding and sculpting the mitochondrial membrane.

To study the roles of BAR domain in membrane binding, a BAR domain construct without the C-terminus of FAM92A1 (FAM92A1 BAR domain) was constructed (**I, Figure 4A**). The purified FAM92A1 BAR domain protein exists mainly as dimers with a few oligomers, displaying the canonical character of the BAR domains (Gallop and McMahon 2015; Mim et al. 2012) (**I, Figure 4B**). Compared with full-length protein, the BAR domain mutant displayed a reduced membrane binding affinity to both the MOM and MIM in vesicle co-sedimentation assays (**I, Figure 4C**). In addition, a construct without the N-terminal putative mitochondrial targeting sequences named FAM92A1 $\Delta_{1-40aa}$  was cloned for mimicking the endogenous FAM92A1 (**I, Figure 4A**). The results revealed that FAM92A1 $\Delta_{1-40aa}$  preserved membrane binding ability to the MOM and MIM, but with a lower affinity than the full-length protein (**I, Figure 4C**).

#### **13.2 FAM92A1 preferentially binds to negatively charged phospholipids**

To address the lipid-binding specificity of FAM92A1, vesicles containing different lipid compositions were used in membrane binding assays. The full-length FAM92A1 and mutants bound to the phosphatidylcholine (PC): phosphatidylethanolamine (PE) membranes. Interestingly, the negatively charged phospholipids, phosphatidylinositol 4, 5-bisphosphate (PI(4,5)P<sub>2</sub>) or cardiolipin significantly augmented the membrane binding ability of both full-length FAM92A1 and mutants (**I, Figure 4D**), suggesting that FAM92A1 preferentially binds to the negatively charged lipids. PC and PE are the major phospholipid classes in eukaryotic mitochondria, but the main difference of lipid composition between the MOM and MIM is the ratio of PI and cardiolipin. PI is present at large amount in the MOM and cardiolipin is enriched in MIM with 18% (Horvath and Daum 2013; WATT et al. 2002). Thus, the full-length FAM92A1 and mutants showed a similar binding ability to vesicles containing phospholipid compositions of the MOM and MIM. These data verified that FAM92A1 binds to PI(4,5)P<sub>2</sub> and cardiolipin with a similar affinity, as shown in **I, Figure 4C**. This finding is in line with the characteristic binding preference for negatively charged phospholipids shared by all BAR domain proteins (McMahon and Boucrot 2015).

#### **13.3 FAM92A1 sculpts membranes into tubular structure**

Having demonstrated that FAM92A1 binds to mitochondrial model membrane, we asked whether FAM92A1, like other BAR domain proteins, can directly sculpt the membrane structure. Strikingly, the full-length FAM92A1 and its BAR domain transformed spherical liposome into narrow tubule (**I, Figure 4F**), suggesting that FAM92A1 possesses membrane-remodeling activity by generating positive membrane curvature. Similar to the diameter of membrane tubule induced by classical BAR

domain proteins, the average diameter of tubules sculpted by FAM92A1 full-length and BAR domain proteins was around 32 nm and 34 nm, respectively (**I, Figure 4H**). Interestingly, different from the wild-type and BAR domain proteins, the diameter of membrane tubule generated by FAM92A1 $\Delta_{1-40aa}$ , a mutant protein with the N-terminal 40 amino acids cleaved off, were narrower than those sculpted by the full-length and BAR domain proteins. The average diameter of tubule generated by FAM92A1 $\Delta_{1-40aa}$  was around 20 nm (**I, Figure 4H**). A possible reason for the narrow diameter is that membrane insertion of the N-terminus of full-length FAM92A1 could change the spontaneous membrane curvature, thus regulating the diameter of membrane tubule (Boucrot et al. 2012; Campelo, McMahon, and Kozlov 2008; Gallop et al. 2006; Saarikangas et al. 2009).

Taken together, these results reveal that FAM92A1 displays membrane-remodeling activity by interacting with the mitochondrial membrane and generating positive membrane curvature, indicating the key function of FAM92A1 in regulating mitochondrial membrane ultrastructure.

#### 14. Membrane binding interface of FAM92A1

Numerous studies have shown that negatively charged phospholipids produce a negative electrostatic potential, which attracts clusters of positively charged residues on proteins. These electrostatic interactions mediate the anchor of proteins to lipid membrane (Aponte-Santamaria et al. 2012; McLaughlin 2005; Zimmerberg and McLaughlin 2004). As an important factor in membrane-remodeling processes, the BAR domain superfamily has a membrane binding interface on which clusters of positively charged amino acids are positioned to interact with negatively charged phospholipid headgroups of lipid membranes (Frost et al. 2008). In *Drosophila*, the BAR domain protein amphiphysin binds to highly curved negatively charged membranes and drives membrane curvature through its N-terminal amphipathic helix (Arkhipov, Yin, and Schulten 2009; Peter et al. 2004). Mutation of positively charged residues (lysine and arginine) to negatively charged residues reduced the membrane binding affinity of amphiphysin and inhibited tubulation of liposomes *in vitro* (Zimmerberg and McLaughlin 2004).

Similar to the classical BAR domain proteins, FAM92A1 preferentially interacted with the negatively charged phospholipids (**I, Figure 4D**), and thus, we asked whether FAM92A1 possesses positively charged clusters that mediate the membrane association. Sequence analysis found FAM92A1 has seven possible positively charged clusters and to examine whether these clusters are involved in membrane interaction, we mutated the positively charged amino acids arginine (R) and lysine (K) to alanine (A) (**I, Figure 5A**). All mutants were purified for the membrane binding and tubulation assays. As shown in **Figure 5B** of part I, three mutants, Mut3, Mut5, and Mut7, had significantly impaired lipid binding activity compared with the wild-type FAM92A1, suggesting that these three positively charged clusters are involved in the formation of interface when interact with membrane. Furthermore, loss of membrane binding ability profoundly reduced the membrane remodeling activity (**I, Figure 5C and 5D**). Taken together, these data demonstrate that these three positively charged clusters, two residing in the BAR domain module and one at the C-terminal region, are the FAM92A1-membrane association interface.

## 15. Cellular function of GTPBP8

### 15.1 GTPBP8 depletion causes defects in mitochondrial translation

Similar to the nuclear-encoded gene expression process, the process of mtDNA-encoded gene expression can be regulated at the levels of mtDNA stability, transcription, and translation (Christian and Spremulli 2012b; Mai, Chrzanowska-Lightowlers, and Lightowlers 2017; Schatz and Mason 2003). To elucidate the processes involved in the reduced level of mitochondrial-encoded proteins, mtDNA stability was assessed by measuring its abundance in control and GTPBP8-deficient cells using Real-time quantitative PCR (RT-qPCR). As shown in **Figure 3C** of Study **III**, after normalization to the  $\beta$ -globin gene, no significant differences in mtDNA level between the GTPBP8-deficient cells and control cells were found. Moreover, the levels of the mtDNA-encoded mRNA (**III, Figure 3B**) and rRNA (**III, Figure 6D**) were unaffected by depletion of GTPBP8. These data suggest that the reduction of mtDNA-encoded protein abundance was attributed to the mitochondrial translation process, not to mtDNA or RNA levels.

Thus, pulse-labeling experiments were applied to detect the synthesis rate of mtDNA-encoded proteins. After metabolically pulse-labeled with [ $^{35}$ S] methionine/cysteine in the presence of anisomycin, a cytosolic protein synthesis inhibitor, the results showed a clear synthesis defect for the majority of mitochondrial polypeptides in GTPBP8-knockdown cells compared with control cells (**III, Figure 3D**). Together, these data suggest that GTPBP8 plays a role in the mitochondrial translation process.

### 15.2 GTPBP8 specifically interacts with the large subunit of human mitoribosomes

Mammalian mitoribosome is a complex molecular machinery that sediments as 55S particles, comprising a 28S mt-SSU and a 39S mt-LSU (Christian and Spremulli 2012a; O'Brien, O'Brien, and Norman 2005). Similar to cytosolic ribosomes which are responsible for the synthesis of nuclear-encoded proteins, mitochondrial ribosome is responsible for the synthesis of 13 mtDNA-encoded proteins. Mammalian mitochondrial translation includes initiation, elongation, termination, and ribosome recycling (Mai et al. 2017). GTPBP8 shares sequence homology with the YsxC protein, which is associated with the assembly of the large subunit of the bacterial ribosome (Ruzheinikov et al. 2004; Schaefer et al. 2006; Wicker-Planquart et al. 2008). Thus, to investigate whether the human homolog GTPBP8 plays a similar role in mitochondrial translation, the association of GTPBP8 with the large subunit of human mitoribosomes was examined by using sucrose gradient sedimentation. The mitochondrial lysates were separated on 10-30% continuously sucrose gradients and the results showed that GTPBP8 co-sedimented with mt-LSU proteins. Incubation of the mitochondrial lysates with RNase A, which partially disrupts mitoribosomal integrity prior to loading the sucrose gradient, resulted in GTPBP8 sedimenting in the lighter fractions (**III, Figure 4B**). These results suggest that GTPBP8 is associated with the mitoribosomal large subunit, which may play a role in its assembly and/or stability. More experiments are required to examine whether GTPBP8 is involved in facilitating assembly of the mitoribosomal large subunit and even its interaction partners.

### 15.3 GTPBP8 deletion causes abnormal accumulation of mt-LSU and reduction of mature mitoribosomes

Besides the 13 mtDNA-encoded proteins, mtDNA also encodes 12S rRNA and 16S rRNA (Greber

and Ban 2016). To determine whether the low abundance of mtDNA-encoded mitochondrial proteins were caused by the instability or the degradation of rRNA, the transcriptional level of 12S and 16S rRNA was examined with RT-qPCR. The results showed no obvious change of rRNA profile in GTPBP8-deficient cells in comparison with control cells (**III, Figure 6D**). To examine whether the translation deficiency was caused by the defects in the process of mitoribosomal biogenesis, the gradient sedimentation was applied to analyze the profile of mitoribosomes. The results showed an aberrant profile of mt-LSU in GTPBP8-depleted cells displaying with subunits of mt-LSU (uL11m, and uL16m), but no marked change in the profile of mt-SSU, except for a decreased level of monosomes (**III, Figure 7A**), suggesting that loss of GTPBP8 impaired the assembly process of mitoribosomal large subunit. Collectively, our findings uncover the cellular role of GTPBP8 in regulation of mitoribosomal biogenesis.

## **16. Prickly nanoparticles efficiently inhibit malignant growth of cancer cells via mechanical damage to mitochondria**

Cancer is a complex group of diseases and a leading cause of death worldwide. Despite abundant efforts to find a cure, cancer remains a challenge for modern medicine and many patients continue to suffer from the disease. The field of nanomedicine uses nano-sized tools for the diagnosis, prevention, and treatment of diseases as cancer. In the past several decades, more and more nanoparticles have been synthesized to combat cancer. Nanoparticles are often used as a drug delivery vehicle, selectively delivering drugs to the corresponding cancer tissue (Ferrari 2005). Recently, several studies have synthesized nanoparticles that have led to cancer cell death in the absence of any anticancer drug (Guo et al. 2015; Lukianova-Hleb et al. 2016). For instance, plasmonic nanobubbles can kill cancer cells through laser-induced photothermal therapy (Lukianova-Hleb et al. 2016).

One of our collaboration studies designed and synthesized prickly Zn-CuO nanoparticles (prickly NPs) for anticancer treatment. Prickly@SpAcDX-PEG-VD1142 profoundly inhibit cell viability of hypoxia MCF7 cells (**II, Figure 4**). After escaping from the endosome, prickly NPs mainly target and accumulate in mitochondria (**II, Figure 6**). The accumulation of prickly NPs in mitochondria damages mitochondrial membranes and leads to obvious changes in mitochondrial networks, displaying a swollen and fragmented structure, not the classical tubular structure (**II, Figure 7**). This physical disruption eventually activates apoptosis and causes cell death (**II, Figure 8**). Taken together, mitochondria are promising targets for cancer therapy.

## FINAL REMARKS AND FUTURE PERSPECTIVES

This work systematically elucidated the sub-mitochondrial localization, mitochondrial import and biological functions of two novel mitochondrial proteins using the combined approaches of biochemistry, cell biology and molecular biology. Through studying the function of these proteins, we noticed that the normal mitochondrial morphology, function, and dynamics are essential for cell homeostasis. Dysfunction of essential mitochondrial proteins is linked to many human diseases. A previous study found that high expression of FAM92A1 accelerates cell proliferation and tumorigenesis, highlighting FAM92A1 as a potential oncogene (Liang et al. 2009). However, how FAM92A1 modifies the cell cycle and the molecular mechanism underlying tumorigenesis were poorly understood. In addition, full-length FAM92A1 protein contains one BAR domain at the N-terminus and a short C-terminus (**I, Figure S1A**). The classic BAR domain proteins sculpt membrane remodeling with actin filaments that function in endocytic pathways and filopodia formation (Ahmed et al. 2010).

Different from the classic BAR domain proteins, FAM92A1 localizes to the mitochondrial inner membrane facing the matrix. The cell growth arrest of FAM92A1-depleted cells was caused by impairment of mitochondrial ultrastructure. Disruption of the mitochondrial inner membrane ultrastructure, which is the site for the oxidative phosphorylation complexes, affects the complex assembly, electron transportation and ATP production, resulting in cell growth arrest. Whether FAM92A1 interacts with other mitochondrial proteins that sculpt the mitochondrial membrane is unknown. Light should also be shed on how the MICOS complex is reduced in the FAM92A1-depleted cells.

In addition, disturbance of the mitochondrial membrane structure through physical methods, such as prickly NPs, caused the release of pro-apoptotic proteins from the intracristal spaces to cytosols. Release of pro-apoptotic proteins and activation of apoptotic proteins, a classical mitochondrial-mediated apoptotic pathway, caused cancer cell death.

Mitochondrial structure is important for cell activity and energy generation also controls the cell fate. The assembly of oxidative complexes maintains cell energy requirements. Thirteen mitochondrial-encoded proteins are essential subunits of all OXPHOS complexes, except complex II. Therefore, any process affecting the synthesis of mtDNA-encoded proteins can induce energy shortage. In our study, GTP-binding protein 8 was associated with the mitochondrial ribosomal large subunit. Loss of GTPBP8 caused an aberrant profile of large subunits and also reduced the level of monosomes. Abnormal assembly of mitochondrial ribosomes caused by depletion of GTPBP8 inhibits the synthesis of mitochondrial-encoded proteins, thus leading to mild defects in assembly of OXPHOS complexes. These defects reduced mitochondrial respiration and ATP production. In summary, elucidating the molecular basis of mitochondrial proteins is crucial for understanding mitochondrial structure and function.



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